Day length and growth

Boon, Polly Ester

DAY LENGTH AND GROWTH

BEHAVIOUR – ENERGY BALANCE – PROTEIN SYNTHESIS

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RIJKSUNIVERSITEIT GRONINGEN

DAY LENGTH AND GROWTH

BEHAVIOUR – ENERGY BALANCE – PROTEIN SYNTHESIS

Proefschrift

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te 's Gravenhage

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Referent: Dr. G.H. Visser

Als men die oceaan van kennis bevaart, bereikt men nooit de kust.

(Chinees spreekwoord)

CONTENTS

Chapter 1	General introduction	9
Chapter 2	Effect of photoperiod on body mass, and daily energy intake and energy expenditure in young rats *P. Boon, G.H. Visser, S. Daan*	15
Chapter 3	Effect of day length and food duration on body mass, and daily energy intake and energy expenditure in Japanese quail (*Coturnix c. japonica*) chicks *P. Boon, G.H. Visser, S. Daan*	27
Chapter 4	Effect of short daily feeding periods on body composition in Japanese quail (*Coturnix c. japonica*) chicks *P. Boon, I. Everts, G.H. Visser*	51
Chapter 5	Feeding and body mass of Japanese quail (*Coturnix c. japonica*) chicks with unpredictable food access *P. Boon, G.H. Visser, S. Daan*	65
Chapter 6	Effect of day length on the response of protein synthesis to fasting and feeding in growing Japanese quail (*Coturnix c. japonica*) chicks *P. Boon, P.W. Watt, G.H. Visser*	79
Chapter 7	Validation of the doubly labelled water method in Japanese quail (*Coturnix c. japonica*) chicks: is there an effect of growth rate? *G.H. Visser, P. Boon, H.A.J. Meijer*	91
References		105
Samenvatting		117
Nawoord		123

CHAPTER 1

GENERAL INTRODUCTION

Day length affects growth rate in young birds and mammals. Like most animals these homeotherms are usually either diurnal or nocturnal. This means that their basic pattern of behaviour has evolved such that activity coincides with either day or night. A whole suite of behavioural, morphological, and physiological adaptations supports this specialisation in a temporal niche. The metabolic rate, for instance, varies in the course of the light–dark cycle with a maximum during the favoured part of the cycle: the day in diurnal, and the night in nocturnal species. For a growing animal this may have important consequences. The length of day and night, as it varies with season and latitude, determines the duration of activity, and hence 24-h energy intake and 24-h energy expenditure. Growth can be viewed as the resultant of these last two processes. In diurnal animals, longer day lengths create more opportunities for energy intake that should stimulate growth. Longer day lengths also increase energy expenditure by creating more opportunities for locomotor and feeding activities that should in turn reduce weight gain. Shorter day lengths have the reverse effect of reducing both energy intake and energy expenditure. This interaction between energy intake and energy needed for activities (and thus lost for growth) as a function of day length determines the overall weight gain (Fig. 1).

Several reports have demonstrated effects of day length on juvenile growth rates (84,124,125,144,162,175,185). Such effects may have important implications for our understanding of life history adaptations in animals breeding at different latitudes (153) or at different times of the year (37). They also have consequences for the optimisation of day length scheduling in animal husbandry. It is therefore important to understand the pathways – behavioural and physiological – along which day length affects energy balance, and thus growth. These pathways are not passive responses to the duration of light (118). When exposed to short day lengths, diurnal animals are able to adjust both feeding and activity levels in such a way that high growth rates are maintained despite limitations in food supply. Other compensatory mechanisms ensuring high growth rates under shorter day lengths are adjustments in food digestion. Such adjustments can be achieved by preferential allocation of energy to the development of digestive organs, and/or via an improved balance between food intake and digestion. I will briefly review the current state of knowledge on these adaptive mechanisms, and will thereby focus on diurnal species.

Adjustment of feeding and activity to day length

Growing animals subjected to decreasing day lengths can increase their rate of energy intake during the time food is available, and thus increase their total 24-h energy intake. This increase in feeding rates can be achieved by enlarging (external or internal) hoarding. Birds may hoard food internally in their crop (4,9,24,70,142,180) or oesophagus (76). Hamsters are known to hoard food externally in their burrows when subjected to food restriction (7,137,196). Such hoarding activities permit young animals to consume more food than expected on the basis of day length reduction. In this way they manage to tide over prolonged daily fasting (= dark) periods and to grow continuously.

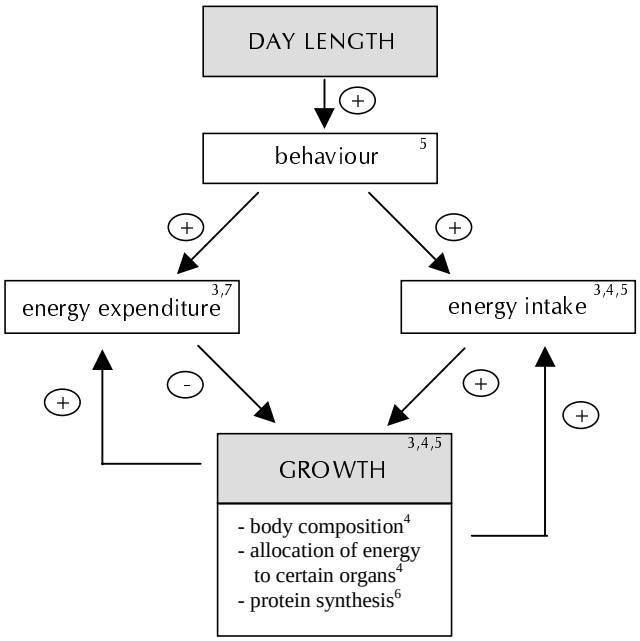

Figure 1. Schematic view of the interaction between day length and growth in a diurnal species. Numbers refer to chapter numbers.

Another behavioural adjustment that may allow animals to grow optimally despite decreasing day lengths is a reduction in activity during the time no food is available. This reduction leads to a decrease in overall 24-h energy expenditure, so that more energy is available for growth. For example, growing chickens and laying hens were able to decrease energy expenditure during a 10-h dark period by 22% as compared to only 12% of the light period value when exposed to a daily 1-h dark period (94). In adult pigeons prolonged (nocturnal) fasting periods led to a pronounced lowering of energy expenditure during the night, resulting in lower 24-h energy expenditure levels (145).

Adjustments of energy allocation to day length

A decrease in day length can also induce adjustments of the digestive organs that may increase feeding efficiency and/or food storage capacity, and thus ensure continuous growth (4,73,74,140). It has been hypothesised that young birds facing an environmental challenge, such as a reduction in day length, may exhibit compensatory changes in rates of metabolism and/or maturation (163). In this way chicks would be able to grow at maximum rate. Extending this hypothesis to include the gastrointestinal tract would imply that by morphological adjustments of the

digestive organs chicks may increase their feeding efficiency and/or food storage capacity, and thus ensure maximal growth rates.

A decrease in day length may further increase feeding efficiency (and thus growth) via an effect on the balance between food intake and digestion (28). Throughout the day food intake occurs, whereas during the night, when both food intake and activity are absent or suppressed, digestion can take place. Certain light–dark ratios within a 24-h period may improve this balance between food intake and assimilation, resulting in higher weight gains. Experiments in poultry have shown that intermittent lighting (for example 2L:12D:2L:8D) has a positive effect on feeding efficiency (30,127,159,168,169) with improved body mass gain (30,127,168).

All these compensatory mechanisms may contribute to the maintenance of high growth rates in growing juvenile homeotherms when subjected to decreasing day lengths. Under extreme short day lengths however, the daily feeding period is bound to become too short to compensate for a reduction in energy intake by adjustments of energy expenditure and/or allocation. Growth rates will then be reduced with negative consequences for survival rate and future reproduction (22,65). Theoretically, this also holds for extreme long day lengths. When day length increases, the rise in energy intake will initially offset the detrimental effect of higher energy expenditure levels on growth: growth rates increase. However, when day length is increased further, eventually a point is reached where energy intake is at its maximum rate and only energy expenditure will increase further: growth rates decrease.

Evidently day length influences growth rates in many different ways. The interplay of these factors determines the overall weight gain animals are able to achieve when subjected to a certain day length. The aim of the experiments discussed in this thesis is to investigate the role of day length in determining overall growth in juvenile animals (Fig. 1). To study this we chose Japanese quail (*Coturnix c. japonica*), a diurnal species with the highest growth rate in the family Phasianidae (151), and therefore likely to be responsive to manipulations in day length. We studied two strains to compare breeds with different growth strategies resulting from selective breeding. We investigated the effect of day length on different parameters: energy intake, energy expenditure, locomotor activity, feeding and drinking behaviour, body composition, and protein synthesis. For comparative reasons, we also conducted an experiment in which we studied the effect of day length on growth in a nocturnal species, the rat. We chose for the rat, because its weight gain is known to respond to manipulations of the light–dark cycle (95,158,182). Furthermore, the rat – as a mammal – has a lower growth rate, and a lower metabolic rate compared to birds of equal mass.

In chapter 2 we begin our research with an experiment on the effect of either a long (18L:6D) or short day length (6L:18D) on locomotor activity, body mass, growth efficiency (relationship between body mass change and food intake), energy intake, energy expenditure, and body composition in growing Wistar rats during a period of 190 d after weaning. This chapter addresses the question whether day length generates differences in weight increase by affecting energy intake and/or energy expenditure. Results showed that the rat, a nocturnal species, has a higher growth rate

when subjected to long day lengths, due to the effect of day length on the distribution of energy intake and energy expenditure over the total 24-h cycle.

Chapter 3 describes an experiment on the effect of different day lengths (18L:6D, 15L:9D, 12L:12D, 9L:15D, and 6L:18D) on body mass increase, and 24-h energy intake and energy expenditure in two strains of Japanese quail (7–71 d of age), a fast growing strain bred for meat production and a normal growing strain bred for commercial egg production. Food was only available during the light period. In this chapter we evaluate how day length and food availability contribute to the overall energy balance. The experiment showed that changes in feeding behaviour and energy expenditure in response to day length and food availability manipulations are part of a general strategy in chicks to maximise energy intake and minimise energy expenditure. This strategy enables chicks to gain body mass continuously.

Results, as described in chapter 3, reveal that a reduction in day length (and hence food availability) led to behavioural adjustments in both feeding and activity levels in young quail. These adaptations allowed them to grow continuously despite limitations in food supply. Apart from these behavioural adjustments, food restriction (via day length manipulations) may also lead to morphological adaptations of the digestive organs. This is addressed in chapter 4, where we studied the effect of day length at organ level in the fast growing strain of Japanese quail (7–28 d of age) subjected to either a long (18L:6D) or short day length (6L:18D) with *ad lib.* food during the light period. We describe the development of heart, pectoralis muscle, liver, different components of the gastrointestinal tract, and spleen. Results showed that young quail increase food hoarding capacities (by enlarging crop mass) when confronted with shorter day lengths (and hence shorter feeding periods). In this way they increase energy intake to a level that enables them to grow continuously.

Chapter 3 and 4 show that young quail subjected to a 6-h day length learned to exploit their crop as a temporary storage place for food. This could be mobilised when no food is available, and enabled chicks to grow continuously. Chapter 5 describes an experiment into the motivation of these birds to fill their crop in anticipation of a prolonged fast. To this end, we subjected fast growing Japanese quail (7–31 d of age) to a long day length (18L:6D) with short (6 h) daily feeding periods with unpredictable feeding times. The effects on locomotor activity, feeding and drinking behaviour, energy intake, and body mass were studied. Results revealed that young quail seem to adjust their feeding behaviour in response to their instantaneous energy needs. This effect is modulated by the time of food arrival: food arriving later leads to higher intake levels.

Growth in young animals is mainly growth in protein, achieved by maintaining protein synthesis at a higher rate than protein breakdown (188). As shown in chapter 3 and 4, day length (and consequently food availability) influences overall growth. It seems therefore feasible that day length also affects protein turnover by influencing protein synthesis and/or protein breakdown rates, and thus net growth. Hence, in chapter 6 we investigate the effect of day length on the response of protein synthesis rates to fasting and feeding. For this, Japanese quail of the fast growing strain (7–21 d of age) were subjected to either a long (18D:6L) or short day length (6L:18D) with *ad lib.* food during the light period. We measured mixed protein fractional synthesis rate

(% of protein mass synthesised per h) of pectoralis muscle, liver, and heart after an overnight fast and after 2 h food access at dawn. Protein synthesis was measured by a flooding dose of L-[1-^{13}C] labelled leucine. This chapter reveals that day length, by determining the daily period in which feeding can occur, has a major effect on protein synthesis rates. This effect determines the overall growth chicks are able to achieve when exposed to different day lengths.

Finally in chapter 7, the doubly labelled water (DLW) method is validated against respiration gas analysis in growing Japanese quail of both the fast and normal growing strain from 1 week of age to adulthood. The DLW method is a technique for measuring energy expenditure under free-living conditions. Over the years, this method has been extensively validated in adult animals, but not in growing animals. A problem that may arise using the DLW method in growing animals is that isotopes not only leave the body water pool as water or CO_2 gas, but also via incorporation into growing tissues. This may theoretically lead to an underestimation of the CO_2 production rate. This chapter demonstrates that the DLW method is also well suitable to measure energy expenditure in growing birds.

CHAPTER 2

EFFECT OF PHOTOPERIOD ON BODY MASS, AND DAILY ENERGY INTAKE AND ENERGY EXPENDITURE IN YOUNG RATS

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ABSTRACT

In this experiment we investigate the effect of photoperiod on locomotor activity, body mass, food intake, growth efficiency (relationship between body mass change and food intake), energy expenditure, and body composition in growing Wistar rats. Two groups of animals were subjected to either a long, 18L:6D ($n = 8$), or short photoperiod, 6L:18D ($n = 7$), during a period of 190 d after weaning. Activity, body mass, food intake, and energy expenditure were measured during the study, as well as body composition at the end of the experiment. We show that growing rats, exposed to a short photoperiod (a) have a lower rate of weight gain, (b) have similar energy intakes, (c) have lower growth efficiency, (d) have lower daily energy expenditure and resting metabolic rate, and (e) gain less lean body mass than those exposed to a long photoperiod. We suggest that the distribution of energy expenditure and food intake over the total 24-h cycle may be responsible for the difference in body weight gain between the two photoperiods.

INTRODUCTION

Many species of mammals undergo pronounced seasonal fluctuations in body mass, although the direction of these fluctuations varies (61,184). Such fluctuations in adult mammals have been shown to be under the control of day length. Voles, for example, reduce their body mass when subjected to short photoperiods, whereas Syrian hamsters are known to increase their body mass under these circumstances (22,25,38,184). Also the growth rates of juveniles are influenced by photoperiod (124,125,150,185). Growing male collared lemmings exposed to short photoperiods (8L:16D) at weaning show a higher increase in body mass than animals subjected to a long photoperiod (20L:4D; 124). The response of body mass on photoperiod in both adult and juvenile animals is a seasonal adaptation to a changing environment, which has major consequences for survival rate and future reproduction (22,65).

Obese Zucker rats, when exposed to a long photoperiod (14L:10D) for 9 weeks (from 1 week after weaning), increase their body mass more than rats from a short photoperiod (10L:14D), without showing differences in food intake (84). It was hypothesised that a possible factor contributing to the observed effect is a difference in activity level between the two photoperiods (84). By reducing the active (nocturnal) phase, the animals presumably reduce their daily energy expenditure and spend more time resting. Another possible explanation given is a change in metabolic efficiency (84). Rats take their meals mainly during the dark period. Consuming the same amount of energy in a shorter time period could have a positive effect on the feeding efficiency and could therefore result in a larger increase in body mass per gram of food intake (84). In contrast no effect of photoperiod on growth in lean rats was observed (84), which could be due to the minor difference in the length of the light phase (4 h) between the two photoperiods.

Body weight gain is the resultant of daily rates of energy intake and energy expenditure. It is not known for any of the species studied so far whether day length generates differences in weight gain by affecting energy intake and/or energy expenditure. Our study addresses this question. We studied the rate of weight gain of rats exposed to different photoperiods. Rats were chosen because their weight gain responds to other manipulations of the light–dark cycle (95,158,182), although responses to day length appear to be restricted to weight gain in obese Zucker rats (84). We exposed nonobese male Wistar rats to two different photoperiods which differed in day length by 12 h and examined the effect of photoperiod on locomotor activity, body mass, growth efficiency, food intake, energy expenditure, and body composition. We extended the experiment until 190 d after weaning to examine the effect of photoperiod on adult body mass and composition.

METHODS

Animals and housing

Male Wistar rats were randomly assigned to either a long, 18L:6D ($n=8$), or short photoperiod, 6L:18D ($n=7$; lights on at 0930 or 2130 hours MET, respectively), directly after weaning when the animals were 4 weeks of age (= day 0). The animals were housed individually in perspex cages ($l \times b \times h$: 25.5×25.5×30 cm^3) with wood shavings for bedding. The cages were placed in two different light-tight wooden boxes (each with its own photoperiod). The boxes were placed in a dark, sound attenuated room at 25°C and 65% humidity. Every 2–4 d, the animals were weighed at the end of the light episode, and food intake was recorded at the same time. At four time points during the experiment (at day 15, 30, 90 and 160), the distribution of food intake over the light and dark episodes was recorded by weighing the feeder at the start and end of the different episodes.

Food (RMH-B, Hope Farms BV, The Netherlands), a pellet-diet containing 22.8% (w/w) crude protein and 17.7 $kJ \cdot g^{-1}$, and water were freely available. To obtain an expression for the "efficiency" in which food increases body mass, we calculated the change in body mass over 2–4 d and divided this by the amount of food (g) eaten over the same period: the growth efficiency.

Locomotor activity was continuously recorded by passive infrared detectors (PID, Wonderex FX-35) placed above the home cages of seven animals of each group. Movements detected were automatically recorded every 2 min throughout the experiment. For the calculation of the total activity over 24 h and the activity during the light and dark episodes, the 2-min values were added for the different episodes. To calculate the intensity of activity during the light and dark episodes, the total amount of activity per episode was divided by the amount of hours in each episode.

Energy expenditure

We measured daily energy expenditure (DEE) at regular time intervals with indirect calorimetry, in which oxygen consumption and carbon dioxide production are measured in an open air flow system. For this, the animals were removed from their home cages and placed in airtight metabolic boxes of 20 L with wooden shavings for bedding. The boxes were placed in light- and temperature-regulated metabolic chambers. The light schedule and temperature were identical to the conditions in the home cages. The animals were placed in the metabolic boxes during the light period. Metabolism was measured over a period of at least 25 h to obtain a full 24-h record after possible handling and gas equilibration effects had subsided. During the measurement food and water were freely available. Body mass was recorded at the start and end of the measurement.

Dry air was pumped through the boxes at rates varying with age (from ca. 45 $L \cdot h^{-1}$ on day 8 to ca. 120 $L \cdot h^{-1}$ on day 152) to obtain a difference in % oxygen in the in- and outflowing air of about 0.5%. The flow rate was measured on the inlet air with a mass-flow controller (Type 5850E Brooks) to an accuracy of 1%. The excurrent air was dried over molecular sieves (3 Å, Merck). The oxygen concentration in the in- and outflowing air was measured by a zirconium oxide sensor (S-3A/II Oxygen

Analyser, Applied Electrochemistry), and the carbon dioxide concentration by an infrared gas analyser (BINOS-IR), both to an accuracy of 0.01%. We employed six channels simultaneously, using valves to switch between the channels once per minute (washout time 45 s), so that for each animal the values were recorded automatically at 6-min intervals. The system recorded the oxygen and carbon dioxide differentials between dried reference air and dried air from the metabolic box.

We calculated oxygen consumption and carbon dioxide production ($L \cdot h^{-1}$) using Eqn 6 of (63), in which the gas data are corrected for changes in gas volume resulting from the carbon dioxide production with the use of the respiratory quotient (RQ). The obtained values were converted to energy expenditure ($kJ \cdot h^{-1}$) by applying an energy equivalent of 20.1 $kJ \cdot L^{-1}$ O_2 (53). We calculated the average energy expenditure over the last 24 h of the measurement (daily energy expenditure; DEE), as well as over the light (energy expenditure during the light; EEL) and dark phase of the photoperiod (energy expenditure during the dark; EED). Resting metabolic rate (RMR) was calculated as the lowest value of a 30-min running mean over the last 24 h of the measurement.

Body composition

At the end of the experiment (at day 190), the total body water volume (TBW; g) was determined by ^{18}O dilution (166). Six hours before light offset (0930 hours MET), food was removed. Three hours later, the animals were injected intraperitoneally with approximately 0.5 ml of $H_2{}^{18}O$ (91.37 atom %), sufficient to raise the ^{18}O concentration in the body water to 450 parts per million (ppm) excess. The amount of mixture injected was determined by weighing the syringe before and after injection to the nearest 1×10^{-4} g on an analytical balance (Mettler H54, Tiel, The Netherlands). After injection, the rats were returned to their home cages during a period of 1 h to allow for complete equilibration of the injected isotopes with body water. During this interval the animals were not allowed to drink or eat. After 1 h, we anaesthetised the animals lightly with CO_2, cut off 4 mm from the end of the tail, and collected blood in several 25 μl micropipettes, which were flame-sealed immediately. The animals were weighed before taking blood samples.

The isotopic enrichments of the blood samples were determined at the Centre of Isotopic Research, University of Groningen, by means of isotope ratio mass spectrometry (IRMS) and corrected for natural isotopic abundance (background) in body water. TBW (g) was estimated from the dilution space of ^{18}O:

$$\mathrm{TBW} = \frac{d}{\mathrm{MW}} \times 18.02 \times \frac{(F_d - F_a)}{(F_a - F_b)}$$

where d is the dose of $H_2{}^{18}O$ in grams, MW is the molecular weight of $H_2{}^{18}O$, and F_d, F_a, and F_b are the fractions of ^{18}O in the dose and in the blood samples after (F_a) and before (F_b) isotope administration, respectively. We calculated the lean body mass (LBM; g) from the TBW values by assuming that the dilution space of ^{18}O

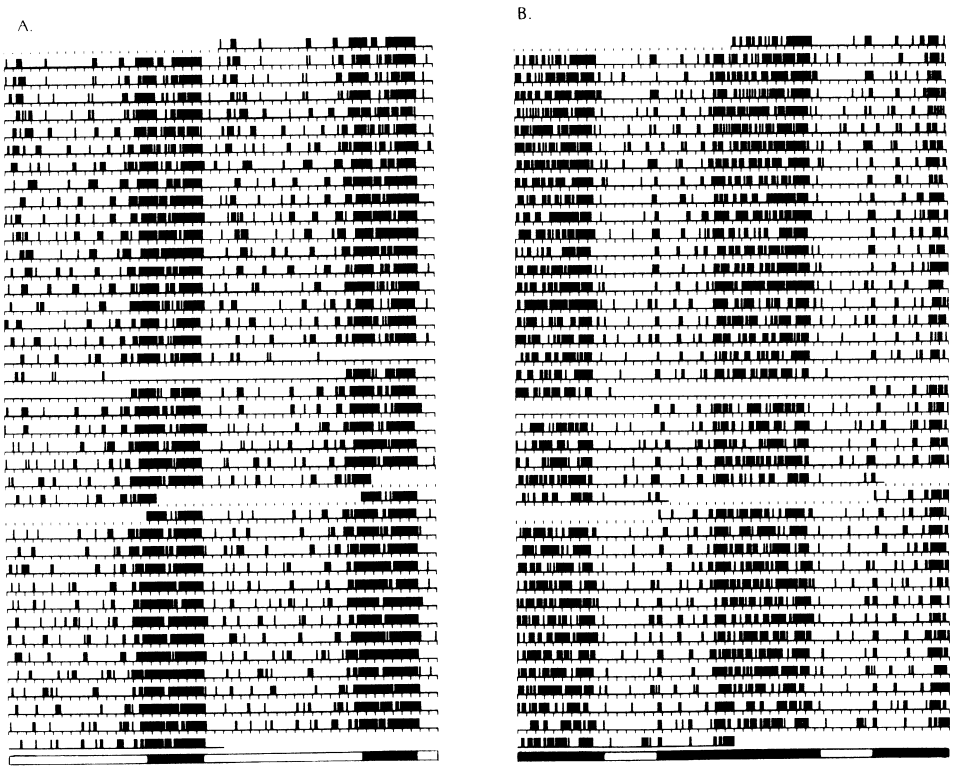

Figure 1. Two representative double-plotted actograms of rats subjected to either a long (18L:6D; A) or short photoperiod (6L:18D; B).

overestimates TBW by 1% (166) and that 73% of the LBM consists of water (100); LBM = (TBW/10.1)×(1/0.73) g. Fat mass (FM; g) was calculated as the difference between body mass and LBM.

Data analysis

Differences between group means were analysed by Student's *t* test (SPSS Inc., 1988). Analysis of covariance (ANCOVA) was used to test for the effects of photoperiod on gross energy intake and energy expenditure after correction for body mass. The repeated-measures procedure was used to test for the effect of photoperiod on growth rate, food intake, and growth efficiency to correct for the dependence of the consecutive measurements on the same animals. All tests were two-tailed, and significance was accepted at $p < 0.05$.

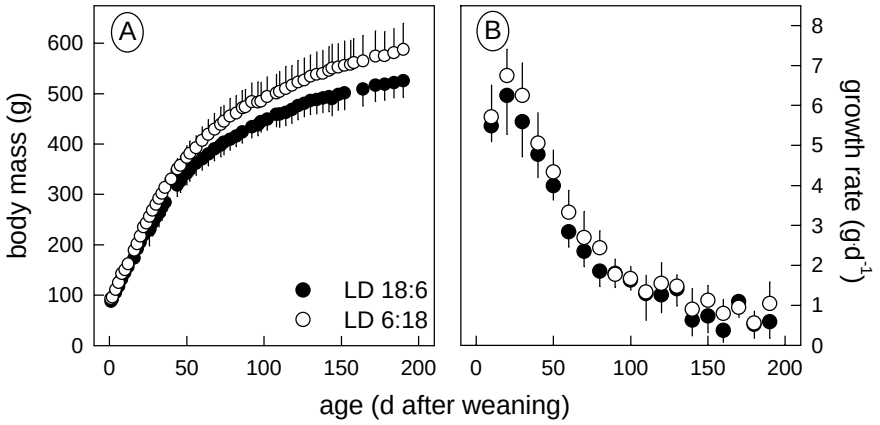

Figure 2. (A) Body mass (g) and (B) growth rate ($g \cdot d^{-1}$), calculated over 10-d periods, as a function of age (d after weaning) in rats subjected to either a long (18L:6D) or short photoperiod (6L:18D). Values are means, and bars indicate SDs.

RESULTS

Locomotor activity

The activity pattern of both groups was highly synchronised with the light–dark cycle (Fig. 1). Most of the activity occurred during the dark, and only incidental bouts of activity were visible during the light phase. Animals subjected to a short photoperiod showed a short bout of activity at the onset of the night. This burst was followed by a 5–6-h episode in which activity was low or absent. Thereafter, a 10–12-h activity bout was visible until about 1 h before lights-on. The animals exposed to a long photoperiod were highly active during the whole night. The start of daily activity occurred about 1–2 h before lights-off and lasted until the end of the night.

The total amount of activity, calculated as the sum of all movements over 24 h, did not differ between the two photoperiods, 6015 ± 1287 movements·d^{-1} for the long-photoperiod group and 7110 ± 647 movements·d^{-1} for the short-photoperiod group. The distribution of the activity was affected by photoperiod. Rats subjected to long photoperiod showed significantly more activity during the light episode, 2629 ± 605 movements as opposed to 610 ± 145 movements for the short-photoperiod group ($T_{6,69} = -8.6$, $p < 0.001$), and significantly less activity during the night, 3386 ± 840 movements and 6501 ± 635 movements, respectively ($T_{12} = 7.8$, $p < 0.001$). The mean level of activity during the light episode was significantly different for the two photoperiods, 146 ± 34 movements·h^{-1} for the long-photoperiod group and 102 ± 24 movements·h^{-1} for the short-photoperiod group ($T_{12} = -2.8$, $p < 0.05$). During the night, the activity level was also significantly higher in animals exposed to a long photoperiod, 564 ± 140 movements·h^{-1} and 361 ± 35 movements·h^{-1}, respectively ($T_{12} = -3.7$, $p < 0.01$).

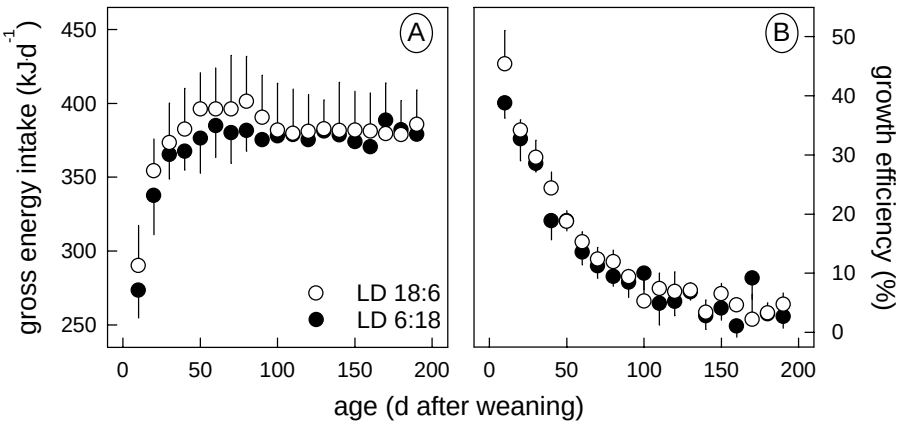

Figure 3. (A) Gross energy intake (kJ·d^{-1}) and (B) growth efficiency (%) calculated over 10-d periods in relation to age (d after weaning) in rats subjected to either a long (18L:6D) or short photoperiod (18L:6D). Values are means, and bars indicate SDs.

Weight gain

At the beginning of the experiment body mass (g) did not differ between the two experimental groups (Fig. 2A). Body mass reached at day 190 was significantly different: 587.6 ± 52.1 g in the long-photoperiod group and 525.3 ± 32.3 g in the short-photoperiod group ($T_{13} = 2.7$, $p < 0.05$). The mean rate of body mass increase over the total experiment was significantly higher in rats subjected to a long photoperiod (2.6 ± 0.2 g·d^{-1}) versus those subjected to a short photoperiod (2.3 ± 0.2 g·d^{-1}; $T_{13} = 2.8$, $p < 0.05$). Growth rate (g·d^{-1}), calculated over 10-d periods, as a function of age is plotted in figure 2B. Growth rate over the first 80 d of the experiment was significantly higher in the long-photoperiod group, 4.6 ± 0.4 g·d^{-1} versus 4.1 ± 0.4 g·d^{-1} for the short-photoperiod group ($F_{1,13} = 7.1$, $p < 0.05$). No difference in growth rate was visible over the last 110 d of the experiment, 1.2 ± 0.2 g·d^{-1} and 1.0 ± 0.2 g·d^{-1}, respectively.

Gross Energy Intake

Gross energy intake (GEI; kJ·d^{-1}), calculated as the mean intake over 10-d periods, is plotted as a function of age in figure 3A. No difference in the mean GEI during both the first 80 d and the last 110 d could be observed between the two groups. The mean GEI over the whole experimental period was not dependent on photoperiod, 379.8 ± 25.5 kJ·d^{-1} for the long-photoperiod group and 367.2 ± 14.6 kJ·d^{-1} for the short-photoperiod group. Correcting GEI for body mass, by incorporating body mass as a covariate in the ANCOVA analyses, did not show an effect of photoperiod on GEI.

The distribution of energy intake over the 24-h period was significantly different for the two photoperiods. Rats subjected to a long photoperiod consumed 46.1 ±

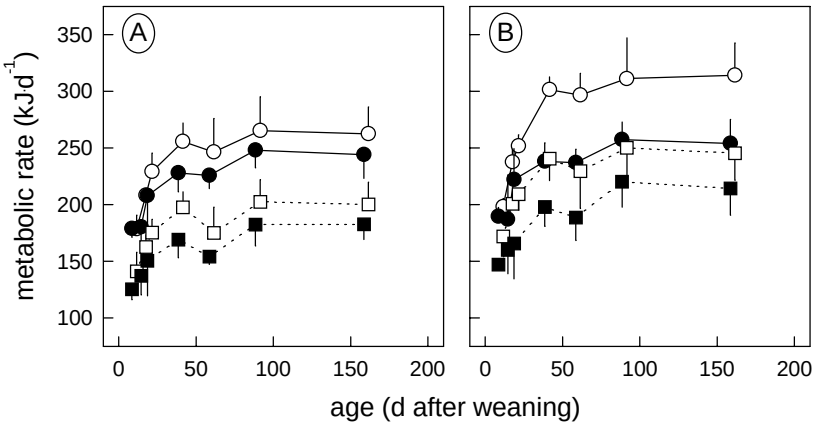

Figure 4. Relationship between metabolic rate (A) daily energy expenditure (circles) and resting metabolic rate (squares); (B) energy expenditure during the dark (circles) and light phase (squares) and age (d after weaning) in rats subjected to either a long (18L:6D; open symbols) or short photoperiod (6L:18D; closed symbols). Values are means, and bars indicate SDs.

5.6% of their daily amount of energy during the light episode, as opposed to only 1.5 ± 2.6% for those subjected to a short photoperiod ($T_{13} = 20$, $p < 0.001$).

The mean growth efficiency (%) over 10-d periods decreased as the animals increased their body mass and became stable around day 100 (Fig. 3B). The efficiency over the first 80 d was significantly higher in the group subjected to a long photoperiod (23.4 ± 1.5%) versus that for the short-photoperiod group (21.4 ± 1.9%; $F_{1,13} = 46$, $p < 0.001$). No difference occurred during the last 110 d of the experiment, 5.8 ± 0.6% and 5.0 ± 1.0%, respectively. The mean efficiency over the total experimental period was significantly higher in rats subjected to long photoperiod, 14.2 ± 1.1% and 12.6 ± 1.6%, respectively ($T_{13} = -2.2$, $p < 0.05$).

Energy Expenditure

In figure 4A,B daily energy expenditure (DEE; $kJ \cdot d^{-1}$), resting metabolic rate (RMR; $kJ \cdot d^{-1}$), energy expenditure during the light (EEL; $kJ \cdot d^{-1}$), and dark (EED; $kJ \cdot d^{-1}$) phase are plotted against age. Both DEE ($F_{1,67} = 6.5$, $p < 0.05$) and RMR ($F_{1,67} = 14.8$, $p < 0.01$) were affected by photoperiod after incorporation of body mass as a covariate in the ANCOVA analyses, with higher rates in the group subjected to a long photoperiod (Fig. 4A). Photoperiod, after correction for body mass, also significantly influenced EEL ($F_{1,67} = 28$, $p < 0.01$) and EED ($F_{1,67} = 67$, $p < 0.01$; Fig. 4B). For none of the energy variables was the interaction between experimental condition and body mass significant.

To examine if the effect of photoperiod on DEE could be attributed to the effect found on RMR, we incorporated RMR as a covariate in the ANCOVA analyses for DEE and found that the effect of photoperiod on DEE disappeared.

Body composition

Table 1. Body composition† (mean ± SD) at 190 d after weaning in rats exposed either to a long (18L:6D) or short photoperiod (6L:18D).

	18L:6D ($n=8$)	6L:18D ($n=8$)
BM‡		
g	581 ± 49	525 ± 34
TBW		
g	346 ± 16*	323 ± 23
%	60 ± 3	61 ± 1
FM		
g	107 ± 33	81 ± 12
%	18 ± 4	15 ± 2
LBM		
g	474 ± 21*	445 ± 31
%	82 ± 4	85 ± 2

† Based on the dilution space of ^{18}O. For more details, see methods section.
‡ BM: body mass; TBW: total body water; FM: fat mass; LBM: lean body mass.
* $p<0.05$.

Based on the dilution space of ^{18}O, the total body water volume (TBW; g) of both groups of animals was estimated, and FM (g) and LBM (g) were calculated. The values are listed in table 1. LBM and TBW were significantly affected by photoperiod ($T_{13}=2.2$, $p<0.05$ and $T_{13}=2.3$, $p<0.005$, respectively), with higher values in the long-photoperiod group. FM and percentage of water, fat, and lean mass did not differ significantly between the two photoperiods.

DISCUSSION

This experiment shows that the rat, which is a nonseasonal breeder and usually not considered to be responsive to day length (186,187), is sensitive to photoperiod during early development. Juvenile Wistar rats exposed to short photoperiods (a) have a lower growth rate, (b) have similar energy intake, (c) have lower growth efficiency, (d) have lower DEE and RMR, and (e) gain less lean body mass than those exposed to long photoperiods. It was also shown that rats exposed to either a long or short photoperiod have no difficulty synchronising their activity pattern to an extreme light–dark cycle.

Extensive studies have shown seasonal cycles of body mass in voles and hamsters that are regulated by photoperiod (6,22,25,38-40,101,184). The reproductive system of these animals is also responsive to photoperiod (5,22,25,38), so that changes in hormone levels can in part be responsible for the observed changes in body mass (27). Other factors that play a role in determining the effect of photoperiod on body mass in these species are changes in food intake (8,39,41,47) and changes in energy efficiency (8). Growth in lean Zucker rats is not responsive to photoperiod, whereas obese Zucker rats do react with higher body masses when subjected to a long photoperiod (84). It was hypothesised that this effect was caused by a reduction in

energy expenditure (extension of the resting phase) and/or by a positive effect of photoperiod on metabolic efficiency by forcing the animals to consume the same amount of energy in a shorter time period (84). The results of our study did not confirm these hypotheses. In growing rats, an extension of the resting (light) phase resulted in an increase in daily energy expenditure, and not a reduction. We did observe a higher growth efficiency in rats subjected to a long photoperiod, as hypothesised in (84). However, we can not conclude from our data that this was caused by a limitation of the feeding time. Rats subjected to a dark period of only 6 h per day learn to consume almost 50% of their 24-h energy intake during the light period.

The effect of photoperiod on daily energy expenditure was explained by a difference in resting metabolic rate (RMR) between the two groups. We calculated RMR as the lowest value of a 30-min running mean over the last 24 h of the energy measurement. This value is dependent on activity level. Although we showed that the total amount of activity did not differ between the two groups, the distribution did. Rats in the long-photoperiod group had a higher level of activity and showed more regular bouts of activity during the light phase than rats subjected to a short photoperiod, which may have prevented energy expenditure from reaching low values. Another possible explanation for higher RMR values in the long-photoperiod group is feeding condition. We showed that rats subjected to long days learn to consume almost 50% of their daily energy intake during the light period, while the short-photoperiod group consumes its energy mainly during the dark period. Although we are not able to say something about the distribution of food intake over the light period (if the animals fast for a couple of hours directly after lights-on or nibble along during the whole light episode), it is possible that during this period the long-photoperiod animals were in a relatively constant feeding condition. They could therefore not reduce their energy expenditure as much as the other group during the light phase and had consequently higher RMR values. Rats that are only fed two meals per day have a lower maintenance energy level than rats that are allowed to eat *ad lib.* (173). The higher values found for energy expenditure during the dark phase in the long-photoperiod group were caused by a higher level of activity exhibited by this group during both phases relative to rats exposed to a short photoperiod. The difference in energy expenditure between the light and dark phases of the photoperiod was larger in the long-photoperiod group than in the short-photoperiod group (Fig. 4B), probably because of the factors mentioned above (feeding condition, activity pattern). Possibly, these fluctuations in energy expenditure over the day had an effect on total energy balance.

The possible difference in food intake pattern, apart from having an effect on the distribution of energy expenditure over the 24 h, could also have influenced growth efficiency. Eating the same amount of energy broadly distributed over 24 h as opposed to a restricted period during the night might have led to differences in growth efficiency. Studies in both rats and humans in which the effect of 'nibbling' (*ad lib.* food intake) and meal feeding (2–4 meals per day) was investigated on growth efficiency have shown no clear effect of feeding pattern (15,132,181,192). However, these studies examined the effect of feeding frequency on growth efficiency in

conditions of weight gain after a period of weight loss (15,192), or in combination with different diets (132), and are therefore not directly comparable with our study. Although rats in the short-photoperiod group did not eat during the light phase, they still had 18 h during the night for feeding. The long-photoperiod group distributed its food intake over the whole 24 h but still ate at the highest rate during the night phase (same amount of energy in 6 h as in 18 h). This situation is not directly comparable with meal feeding and 'nibbling', and therefore an effect of feeding pattern on growth efficiency in our study cannot be excluded.

Another effect of photoperiod in this study is the effect on body composition. Rats subjected to a long photoperiod gained significantly more lean body mass than animals subjected to a short photoperiod. When expressed as a percentage of total body mass, there was no difference. It was previously found that obese Zucker rats exposed to a long photoperiod had a higher lean body mass, and this was attributed to possible altered testosterone levels in the obese Zucker rat (84).

Rats subjected to a long photoperiod from weaning had a higher growth rate relative to rats subjected to a short photoperiod, despite higher energy expenditure levels shown by the first group and the absence of differences in food intake. Growth efficiency was higher in the long-photoperiod group, but it is unlikely that this difference in efficiency accounts for the difference in growth rate between the two photoperiod groups. It seems that the effect of photoperiod on growth in rats is generated by small effects on food intake, growth efficiency, and energy expenditure that accumulate over time and result in differences in body mass gain. The difference in growth rate described here was about 0.4 $g \cdot d^{-1}$ during the first 80 d. If we presume that 1 g of weight increase in young rats consists for 20% of fat and 13% of protein (156), that the synthesis costs of 1 g of fat and 1 g of protein are 39.5 and 23.6 kJ, respectively, and that the efficiency of biosynthesis is 75% (152), the daily difference in energy balance between the two photoperiods would be about 7.5 $kJ \cdot d^{-1}$. This is a marginal difference which will be difficult to verify, but which did, by a cumulative effect, lead to a difference in body mass over time.

In conclusion, the effect of photoperiod on growth in rats is clear, in spite of small differences in growth rate on a daily basis. These differences in body mass gain are most likely produced by differences in the distribution of energy expenditure, and possibly food intake, over the 24-h cycle.

CHAPTER 3

EFFECT OF DAY LENGTH AND FOOD DURATION ON BODY MASS, AND DAILY ENERGY INTAKE AND ENERGY EXPENDITURE IN JAPANESE QUAIL (*COTURNIX C. JAPONICA*) CHICKS

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Submitted

ABSTRACT

Effect of day length and food duration on body mass, energy intake, energy expenditure, respiratory quotient, and sexual development were investigated in two strains of Japanese quail (*Coturnix c. japonica*), a fast growing strain bred for meat production (broilers) and a normal growing strain bred for commercial egg production (layers). In a first experiment we subjected chicks to an 18-h (18L:6D), 15-h (15L:9D), 12-h (12L:12D), 9-h (9L:15D), or 6-h (6L:18D) day length with *ad lib.* food during the light period. In a second experiment birds were exposed to a long day length (either 15 or 18 h) with *ad lib.* food during part of the light period (first 9 or 6 h, respectively). Both experiments were conducted from 7 till 71 d of age. Results revealed that longer day lengths were associated with higher energy intake and energy expenditure levels, resulting in larger weight gains. This effect of day length on weight gain was primarily due to the effect of day length on food availability, and did not differ between strains. The day length below which detrimental effects on weight gain occurred was 9 h. Chicks subjected to day lengths of 9 h or less exploited crop filling to enhance energy intake. They also decreased nocturnal metabolic rates to a greater extent compared to daytime levels than chicks subjected to day lengths of 12 h or more. In addition, these birds showed an anticipatory rise in O_2 consumption prior to feeding, possibly allowing a more efficient use of nutrients during the next feeding period. We also show that sexual maturation was stimulated by day length, and food restriction seemed to delay this process. We conclude that changes in feeding behaviour and energy expenditure shown under short day lengths (and consequently short daily feeding periods) are part of a strategy that allow chicks to gain weight continuously.

INTRODUCTION

Rates of body mass gain in altricial birds in the tropics are on average 23% lower than rates in similar sized birds inhabiting temperate areas (153). A possible explanation for this difference in weight gain rate is a difference in day length between the two latitudes, which determines the period in which parents can gather food for their offspring. Day length is known to have an effect on rates of weight gain in juveniles (19,84,124,125,144,162,175,185). This effect is primarily produced by the effect of day length on both energy intake and energy expenditure (9,19,162). The interaction between these two parameters as a function of day length determines overall body mass gain. In diurnal animals, an increase of day length creates more opportunities for energy intake that should stimulate weight gain. However, longer day lengths also increase energy expenditure by creating more opportunities for locomotor- and feeding activities that should in turn reduce weight gain. Shorter day lengths decrease both energy intake and energy expenditure, because of fewer possibilities for feeding and because energy expenditure is lower at night-time than daytime (2,9,24,57,77,142,145). It is clear that by modulating the duration of the light, and consequently the dark period, opportunities for birds to either increase their food intake or reduce their energy expenditure will vary with consequences for overall body mass gain. Growing chickens and laying hens were able to decrease energy expenditure during a 10-h dark period by 22% as compared to only 12% of the light period value when exposed to a daily 1-h dark period (94). In adult male pigeons (*Columba livia*), prolonged (nocturnal) fasting periods were associated with a high-amplitude cycle in both energy expenditure and body temperature compared to pigeons in *ad lib.* conditions (145). This was due to pronounced lowering of energy metabolism and body temperature during the dark phase, resulting in an overall lower 24-h energy expenditure. Day length can also influence weight gain via an effect on the balance between food intake and digestion (28). Throughout the light period food intake occurs, whereas during the dark period, when both food intake and activity are suppressed, digestion can take place. Certain light–dark ratios within a 24-h period may improve this balance between food intake and assimilation, resulting in higher weight gains. Experiments in poultry have shown that intermittent lighting (for example 2L:12D:2L:8D) has a positive effect on feeding efficiency (30,127,159,168,169) with improved body mass gain (30,127,168). On the other hand, long day lengths stimulate sexual maturation in both quail and other bird species (31,44,78,87,143,169,176,194), which may also affect weight gain positively.

It is evident that daily patterns of food availability and activity have major consequences for the daily amount of food that can be consumed, the total 24-h metabolic rate, and the net balance of energy intake and energy expenditure. In growing animals this balance affects the weight gain animals will be able to accomplish. In this study, we measured in Japanese quail (*Coturnix c. japonica*) the 24-h energy intake and energy expenditure in a range of day lengths with food intake restricted to the light period to evaluate how day length and food availability contribute to energy balance. For this purpose we conducted two experiments. In the first experiment ('day length'), we subjected groups of chicks to five different day

lengths ranging from 6 to 18 h per 24 h with *ad lib.* food during the whole light period. In a second experiment ('food duration'), we studied two groups of chicks that were subjected to a long day length (equal to the longest two day lengths of experiment 1) with only *ad lib.* food during part of the light period (equal to the shortest two day lengths of experiment 1). In this way we could separate the effect of day length from the effect of food duration. We used Japanese quail, a species with the fastest growth rate in the family Phasianidae (151), and likely to be responsive to variations in day length and food availability. Two strains were investigated to compare breeds with different growth strategies resulting from selective breeding. We examined the effects of the experimental conditions on body mass, energy intake, respiratory quotient, as an indicator of metabolic status (9,14,142,145), energy expenditure, and sexual development. We extended the study until adult age to examine the effect of day length on adult body mass.

METHODS

Animals, experimental set up, and housing

Japanese quail (*Coturnix c. japonica*) neonates, of a fast growing strain bred for meat production (broilers) and a normal growing strain bred for commercial egg production (layers), were obtained from a commercial quail farm (N.V. Nouwen, Lommel, Belgium). Until the age of 6 d birds were kept in wooden cages (*l*×*b*×*h*: 67×39×44 cm^3) with sawdust bedding in continuous light and *ad lib.* access to quail starter food and water, to ensure maximum possible body mass gain. A 40 W heating lamp was placed in each cage to provide a temperature gradient sufficient for selection of the preferred temperature by the chicks. At 6 d of age the chicks were assigned to the experimental conditions in such a way that the average body mass did not differ between the groups. In experiment 1 the following day lengths were studied: 18 h (18L:6D; 18LF), 15 h (15L:9D; 15LF), 12 h (12L:12D; 12LF), 9 h (9L:15D; 9LF), and 6 h (6L:18D; 6LF) with *ad lib.* food during the whole light period (Table 1). In experiment 2 groups of chicks were subjected to a long day length (either 15 or 18 h) with only *ad lib.* food during part of the light period (9 or 6 h, respectively); 18L-6F and 15L-9F, respectively. In all groups food became available at lights-on. At 6 d of age, the animals were permitted to habituate to the experimental conditions and allowed to eat *ad lib.* during the whole 24-h period. The experiment started at 7 d of age. Throughout the experimental period a pellet-diet (Institute for Animal Science and Health, ID-DLO, The Netherlands) containing 27.7% (w/w) crude protein and 17 $kJ \cdot wet\ g^{-1}$ (gross energy content as determined by bomb calorimetry; own measurement) was fed. Water was freely available.

During the experiment the animals were housed in pairs in wooden cages (*l*×*b*×*h*: 67×39×44 cm^3) with a wire bottom and a 40 W heating lamp. The heating lamp was gradually raised and finally removed to allow the ambient temperature to decrease to room temperature (~21°C) within 3 weeks of age. One feeder and one water container were mounted on the left and right side of the cages, respectively, and

separated from the inside of the cage by a partition containing two openings, one for each bird. Feeders were automatically removed and returned using a clock-controlled compressed air system. Groups with an uneven number of animals, one cage contained three birds. This had no effect on body mass gain. Birds were sexed by plumage colour.

Body mass and food intake

Birds were weighed (to 0.1 g) every 2–4 d at lights-on till the age of 8 weeks, and thereafter once every week until the end of the experiment. On these days food became available just after weighing. Total 24-h food intake (g) was measured every 2–4 d till the age of 8 weeks, and thereafter once every one or two weeks till the end of the experiment. For this we weighed the feeders at lights-on on two consecutive days. Food intake was measured in two cages in the 6LF, 9LF, and 18L-6F group of both strains, and in four cages in the 12LF, 15LF, 18LF, and 15L-9F group of both strains. Spilled food was carefully collected from all parts of the cage and at the side of the cage where the feeder was located. The 24-h gross energy intake (GEI; $kJ \cdot d^{-1}$) per cage was calculated by transforming 24-h food intake per cage (g) to its energetic equivalent (17 $kJ \cdot g^{-1}$). This value was expressed as GEI per bird.

Energy expenditure

We measured 24-h energy expenditure (EE) at regular time intervals with indirect calorimetry. Oxygen consumption and carbon dioxide production were measured in an open air flow system. The birds were removed from their home cages and placed in airtight metabolic boxes of 10 to 20 L with absorptive paper for bedding. The boxes were placed in light- and temperature-regulated metabolic chambers. The light and feeding schedule, and temperature were identical to conditions in the home cages. The animals were placed in and removed from the metabolic boxes during the light period. We measured metabolism over a period of at least 25 h to obtain a full 24-h record after possible handling and gas equilibration effects had subsided. Metabolism was measured of two animals per box till the age of 4 weeks to minimise the stressful effect of solitary confinement in young birds. Thereafter, the measurements were conducted on isolated animals till the end of the experiment. During the measurement water was freely available. Body mass was recorded at the start and end of the measurement. We performed the measurements every week during the experimental period till the age of 5 weeks. Thereafter, at least one measurement was taken at adult weight. We attempted usually to obtain at least two energy measurements per week and strain for each experimental condition.

Dry air was pumped through the boxes at rates varying with age (from ca. 25 $L \cdot h^{-1}$ at 1 week to ca. 120 $L \cdot h^{-1}$ at 10 weeks of age) to obtain a difference in the in- and outflowing air of about 0.5% oxygen. The flow rate was measured on the inlet air with a mass-flow controller (Type 5850E Brooks) to an accuracy of 1%. The excurrent air was dried over molecular sieves (3 Å, Merck). The oxygen concentration in the in- and outflowing air was measured by a zirconium oxide sensor (S-3A/II Oxygen Analyser, Applied Electrochemistry), and the carbon dioxide concentration by an infrared gas analyser (BINOS-IR), both to an accuracy of 0.01%. At regular time

intervals we calibrated the oxygen and carbon dioxide analysers with certified gas standards. We employed six channels simultaneously, using valves to switch between the channels once per minute (washout time 45 s), so that for each channel the values were recorded automatically at 6-min intervals. The system recorded the oxygen and carbon dioxide differentials between dried reference air and dried air from the metabolic box.

We computed oxygen consumption and carbon dioxide production ($L \cdot h^{-1}$) using Eqn 6 of (63), in which the gas data are corrected for changes in gas volume resulting from the carbon dioxide production with the use of the respiratory quotient (RQ). The obtained values were converted to energy expenditure ($kJ \cdot h^{-1}$) by applying an energy equivalent of 20.1 $kJ \cdot L^{-1}$ O_2 (53). We calculated the average energy expenditure and RQ over the last 24 h of the measurement. The mean RQ was also computed over the last half hour of the dark period for an impression of the metabolic status of the birds at the end of the night. Resting metabolic rate (RMR) was calculated as the lowest value of a 30-min running mean over the last 24 h of the measurement.

Sexual maturity

To assess the effect of day length on sexual maturity, we measured the age and body mass at which females laid their first egg. Because of the experimental set-up (two birds per cage), it was not always possible to identify which bird had laid which egg. In the case of two females per cage, we assumed that the first egg found was laid by the heaviest animal. Because of the characteristic colour pattern of the shells, we could for most eggs establish retrospectively which bird had laid which egg. Eggs were weighed every morning during weekdays just after lights-on.

Data analysis

Data are expressed as means and inter-individual standard deviations. Differences between group means were analysed posthoc by Tukey's "honestly significant difference" test, after an effect of treatment on the variable of interest was ascertained by ONEWAY analysis (SPSS Inc., 1988). Student's *t* test was used when comparing two groups. Because of variations in body mass for the different experimental conditions at the same age, we compared gross energy intake and energy expenditure based on log-log regressions on body mass. To this end, we used analysis of covariance (ANCOVA) with experimental condition and strain as main effects and body mass as covariate. We used the same procedure, but without logarithmic transformation, to test for main effects on other variables with or without body mass as covariate. In all ANCOVA analyses two- and three way interaction terms were taken into account. The main effects and interaction terms were entered in the model. Subsequently we removed the non-significant terms in a stepwise backward procedure. This ANCOVA procedure is an *a posteriori* test without preplanned comparisons and the statistics should be evaluated conservatively (128). Therefore, interaction terms were removed when $p \geq 0.02$ and main effects when $p > 0.05$. A two-tailed significance level of $p < 0.05$ was used in all other tests. More details of the analyses are presented in the results section.

Table 1. Characteristics (mean ± SD) of two strains of Japanese quail subjected to different day lengths and food duration.

	18LF[†]	15LF	12LF	9LF	6LF	15L-9F	18L-6F
Exp. 1						*Exp. 2*	
broilers							
sample size (*n*)	8	9	10	8	8	8	7
females (n)	5	7	5	6	2	6	1
BM start (g)[‡]	38.8 ± 5.5[a¶]	35.3 ± 3.1[a]	33.1 ± 1.3[a]	33.8 ± 4.5[a]	33.1 ± 4.6[a]	33.8 ± 3.6	35.3 ± 5.0
BM increase (g)	262 ± 62[ab]	294 ± 45[a]	261 ± 28[ab]	241 ± 27[ab]	213 ± 25[b]	275 ± 46	204 ± 21
BM gain ($g \cdot d^{-1}$)	4.1 ± 1.0	4.6 ± 0.7	4.1 ± 0.4	3.8 ± 0.4	3.3 ± 0.4	4.3 ± 0.7	3.2 ± 0.7
24-h RQ	0.92 ± 0.05	0.92 ± 0.04	0.88 ± 0.04	0.87 ± 0.05	0.86 ± 0.04	0.89 ± 0.06	0.83 ± 0.03
end of night RQ	0.80 ± 0.06	0.78 ± 0.04	0.73 ± 0.03	0.72 ± 0.03	0.72 ± 0.03	0.76 ± 0.04	0.72 ± 0.03
layers							
sample size (*n*)	13	10	8	7	10	11	15
females (*n*)	4	5	3	3	8	8	11
BM start (g)	27.0 ± 4.2[a]	26.4 ± 2.4[a]	27.1 ± 3.5[a]	23.1 ± 4.6[a]	24.6 ± 1.5[a]	24.4 ± 3.3	26.4 ± 3.3
BM increase (g)	182 ± 53[a]	195 ± 46[a]	187 ± 36[a]	154 ± 14[ab]	131 ± 12[b]	174 ± 31	133 ± 17
BM gain rate ($g \cdot d^{-1}$)	2.8 ± 0.8	3.1 ± 0.7	2.9 ± 0.6	2.4 ± 0.2	2.0 ± 0.2	2.7 ± 0.5	2.1 ± 0.3
24-h RQ	0.94 ± 0.05	0.92 ± 0.05	0.88 ± 0.09	0.88 ± 0.04	0.84 ± 0.02	0.89 ± 0.05	0.84 ± 0.04
end of night RQ	0.78 ± 0.05	0.80 ± 0.05	0.76 ± 0.03	0.73 ± 0.03	0.70 ± 0.02	0.75 ± 0.03	0.74 ± 0.05

[†] For definitions, see methods section.
[‡] BM: body mass; RQ: respiratory quotient.
[¶] Numbers with the same letter within experiment 1 are not significantly (Tukey, $p > 0.05$) different from each other.

RESULTS

Experiment 1: day length

Weight gain

At the age of 6 d body mass (g) did not differ between the experimental groups for both broilers and layers (Table 1, Fig. 1A). On 7 d of age, when food restriction was introduced, the groups subjected to day lengths of 9 h or less did not grow during the first 2 d of the experiment (Fig. 1A). This effect was clearest in the group with a 6-h day length (Fig. 1A). From the age of 9 d onwards weight increased again. Total body mass increase over the whole experimental period in relation to day length is plotted in figure 2A. ANCOVA revealed that the total body mass increase varied significantly with day length ($F_{4,71} = 16$, $p < 0.001$), strain ($F_{1,71} = 138$, $p < 0.001$), and sex ($F_{1,71} = 44$, $p < 0.001$): body weight gain was higher with longer days, in broilers, and in females. Only day length × sex interaction ($F_{4,71} = 8.5$, $p < 0.001$) significantly contributed to the explained variance, indicating that the effect of day length on body weight gain was different for the two sexes: females showed a stronger retardation in body weight increase when day length decreased than males. Adult quail males are known to be smaller than females (13,96). Because of the unbalanced distribution of the sexes over the different groups with few females in the 6LF group of the broilers (Table 1), we only analysed sex differences in total body mass increase. Because sexual dimorphism has been shown to disappear under severe food restriction (52), we do not expect that the small number of females in this group influenced the results.

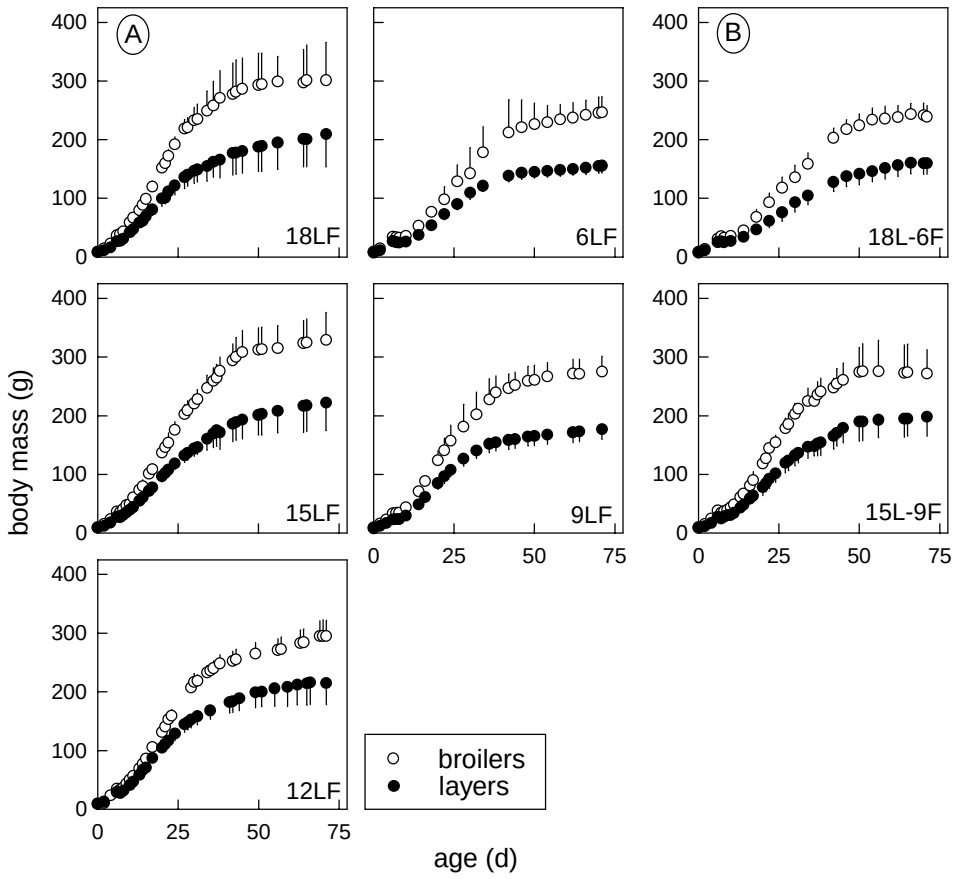


Figure 1. Body mass (g) as a function of age (d) in two strains of Japanese quail subjected to (A) an 18-h (18L:6D; 18LF), 15-h (15L:9D; 15LF), 12-h (12L:12D; 12LF), 9-h (9L:15D; 9LF), or 6-h (6L:18D; 6LF) day length with *ad lib.* food during the whole light period (experiment 1) and (B) long day lengths (15 or 18 h) with *ad lib.* food during part of the light period (first 9 or 6 h, respectively): 15L-9F and 18L-6F, respectively (experiment 2). Values are means, and bars indicate SDs.

Chicks subjected to 9-h day lengths or less started off at lower rates of body mass gain ($g \cdot d^{-1}$) than birds subjected to longer day lengths (Fig. 3A). The 6LF group even lost body mass initially. The rate of body mass gain increased in all groups during the first part of the experiment (Fig. 3A). In the 12LF, 15LF, and 18LF group weight gain rate attained its highest value around 20 d of age, and remained stable from 40 till 71 d of age. In the groups with day lengths of 9 h or less the peak in weight gain seemed to be shifted to around 25 d of age, while it stabilised around the same age (42 d).

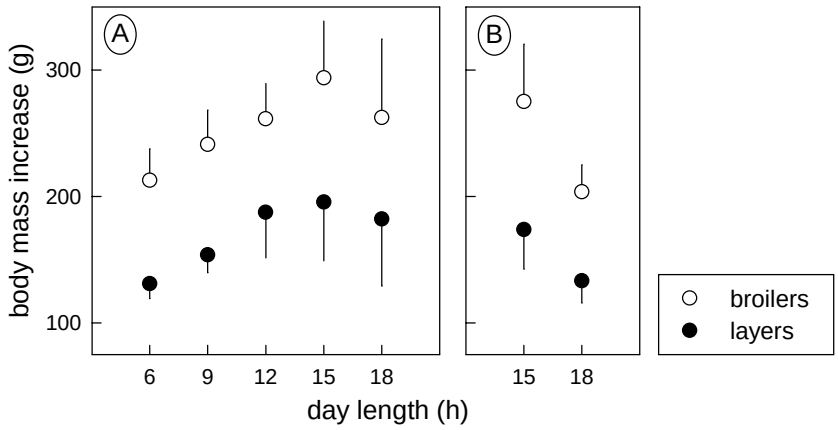


Figure 2. Body mass increase (g) over the whole experimental period in relation to day length (h) in two strains of Japanese quail with *ad lib.* food during (A) the whole light period (experiment 1) and (B) part of a 15-h or 18-h day length (first 9 or 6 h, respectively; experiment 2). Values are means, and bars indicate SDs.

Gross energy intake

The 24-h gross energy intake (GEI; $kJ \cdot d^{-1}$) for the different day lengths is plotted as a function of body mass in figure 4A. For each day length and strain we fitted biphasic regression models to describe the relationship between GEI and body mass (81,83). This was done because of a large increase in GEI for body mass in both the 6LF and 9LF group at the beginning of the experiment (Fig. 4A). The biphasic model is

$$Y = a + b_1 X - r (b_1 - b_2) \log (1 + e ((X - c_1) / r)^{-1})$$

where Y is the log of the dependent variable (GEI), a is the intercept, b_1 en b_2 are the slopes of phase 1 and 2, respectively, X is the log of the independent variable (body mass), c_1 is the estimated breakpoint between phase 1 and 2, and r is a smoothness parameter that was set at 0.01, forcing an abrupt transition (81). All curves were fitted according to the non-linear regression algorithm procedures of the NONLIN package (shareware program, P. H. Sherrod). The significance of adding an additional phase to the model instead of assuming a linear relationship was assessed by an F-test (83). In the 6LF group a biphasic allometric regression model of log GEI with log body mass fitted the data significantly better than a linear regression model in both broilers ($F_{2,32} = 3.6$, $p < 0.05$) and layers ($F_{2,32} = 5.5$, $p < 0.01$). In the 9LF group this was only true for layers ($F_{2,36} = 13$, $p < 0.005$). In all other cases a biphasic pattern did not significantly improve the fit compared to a normal linear regression model ($p > 0.05$; Fig. 4A). We are aware that some birds contribute multiple points to the analyses. However our design does not permit correction for this.

GEI, after correction for body mass, varied significantly with day length ($F_{4,453} = 14$, $p < 0.001$) and strain ($F_{1,453} = 6.0$, $p < 0.025$), with higher intakes at increasing day lengths and in broilers (Fig. 4A). Day length × body mass interaction significantly

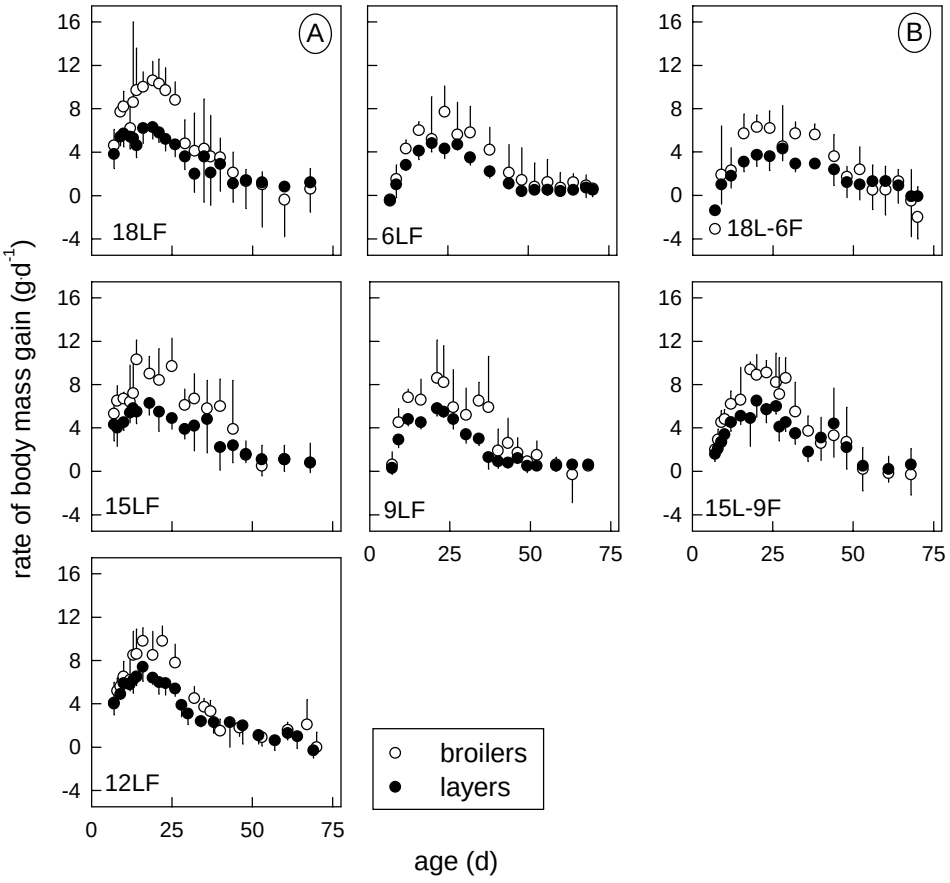
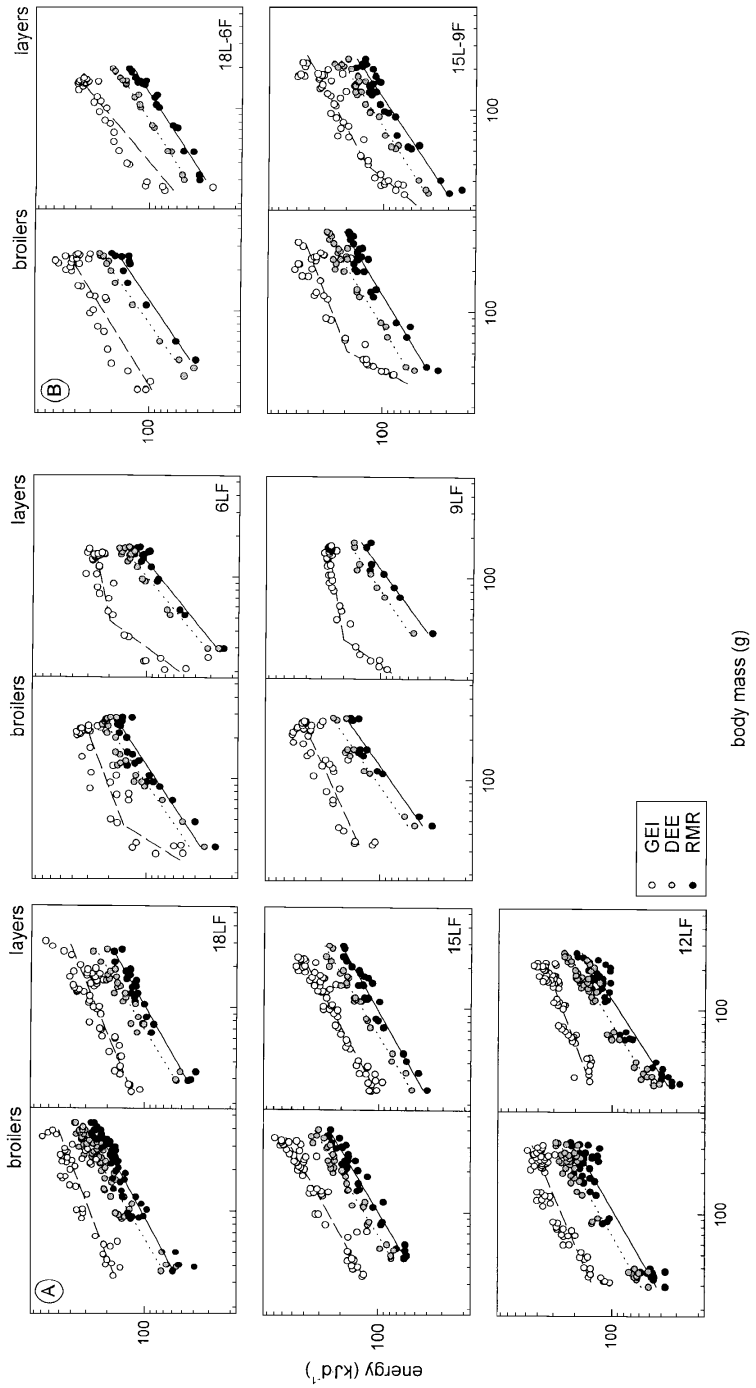


Figure 3. Rate of body mass gain ($g \cdot d^{-1}$) as a function of age (d) in two strains of Japanese quail subjected to different day lengths and food duration. Values are means, and bars indicate SDs. For more details, see figure 1.

contributed to the explained variance ($F_{4,453} = 11$, $p < 0.001$), indicating that the allometric relationship between GEI and body mass differed for the five day lengths: slopes between GEI and body mass were steeper in the groups subjected to day lengths of 9 h or less. For an impression of the GEI levels for the different conditions, we assumed parallel slopes (0.53) for the individual regressions of GEI against body mass by assuming no day length × body mass interaction. GEI level for broilers

→ **Figure 4.** The 24-h gross energy intake (GEI; $kJ \cdot d^{-1}$), 24-h energy expenditure (EE; $kJ \cdot d^{-1}$) and resting metabolic rate (RMR; $kJ \cdot d^{-1}$) in relation to body mass (g) in two strains of Japanese quail subjected to different day lengths and food duration. For more details, see figure 1.


broilers
layers
A
B
18LF
15LF
12LF
18L-6F
15L-9F
6LF
9LF
GEI
DEE
RMR
energy (kJd⁻¹)
body mass (g)
100

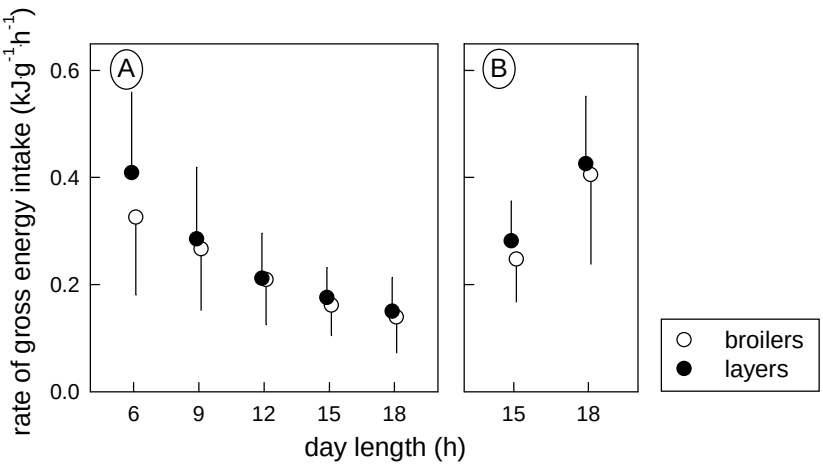


Figure 5. Rate of gross energy intake (kJ·g^{-1}·h^{-1}) as a function of day length (h) in two strains of Japanese quail with *ad lib.* food during (A) the whole light period (experiment 1) and (B) part of a 15-h or 18-h day length (first 9 or 6 h, respectively; experiment 2). Values are means, and bars indicate SDs.

subjected to an 18-h day length was 1.9%, 2.4%, 6.6%, and 29% higher than for chicks of equal body mass in the 15LF, 12LF, 9LF, and 6LF group, respectively. In layers GEI was, for all day lengths, on average 4.7% lower than in broilers of equal body mass.

Figure 5A compares the rate of food intake (kJ·g^{-1}·h^{-1}) between day lengths. The rate was calculated by dividing GEI by its corresponding body mass (Fig. 4A) and the number of feeding hours per day. ANCOVA revealed that feeding rate per g was influenced by day length ($F_{4,454} = 70$, $p < 0.001$) and strain ($F_{1,454} = 5.8$, $p < 0.025$), with higher feeding rates at decreasing day lengths and in layers (9.6%). The interaction term was not significant.

Energy expenditure

In figure 4A we plotted 24-h energy expenditure (EE; kJ·d^{-1}) and resting metabolic rate (RMR; kJ·d^{-1}) as a function of body mass for the different day lengths and strains. We fitted linear regression models, because of no indications that a biphasic pattern between metabolic rate and body mass improved the fit for the different day lengths (Fig. 4A). Also here some birds contribute multiple points to the analyses.

EE ($F_{4,289} = 8.6$, $p < 0.001$) and RMR ($F_{4,289} = 8.0$, $p < 0.001$) varied significantly with day length, after correction for body mass, with higher metabolic rates at increasing day lengths. Strain did not significantly affect EE and RMR. Only day length × body mass interaction was significant for both EE ($F_{4,289} = 5.7$, $p < 0.001$) and RMR ($F_{4,289} = 5.5$, $p < 0.001$), indicating that the slopes of the regressions between metabolic rate and body mass differed between treatments: slopes were steeper at shorter day lengths (Fig. 4A). For an impression of the EE and RMR levels for the different conditions, we assumed parallel slopes (both 0.68) for the individual regressions of EE

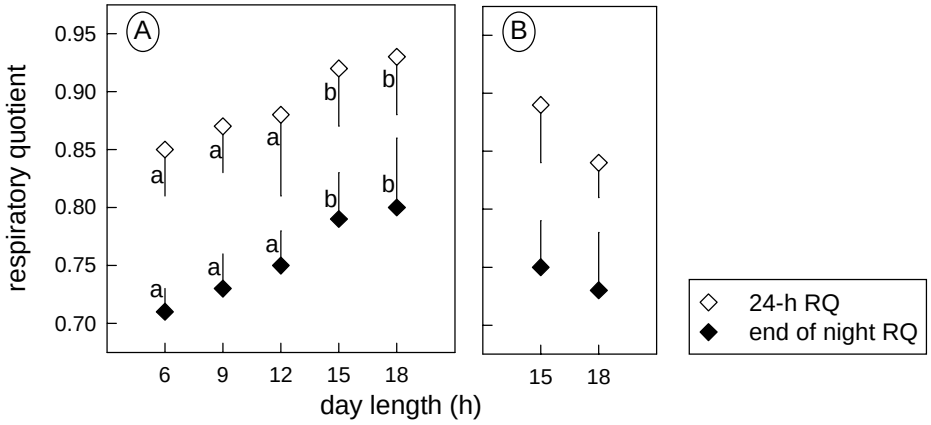


Figure 6. Mean (± SD) 24-h and end of night respiratory quotient (RQ) in relation to day length (h) in Japanese quail with *ad lib.* food during (A) the whole light period (experiment 1) and (B) part of a 15-h or 18-h day length (first 9 or 6 h, respectively; experiment 2). Symbols within variable in left panel (A) with the same number are not significantly (Tukey, $p > 0.05$) different from each other.

and RMR against body mass by assuming no day length × body mass interaction. EE level for 18LF chicks was -0.2%, 9.1%, 14%, and 24% higher than for chicks of equal body mass in the 15LF, 12LF, 9LF, and 6LF group, respectively. The corresponding figures for RMR were -0.4%, 9.5%, 14%, and 20%.

Respiratory quotient

The mean 24-h RQ, after correction for body mass by incorporating this term as a covariate in ANCOVA, varied significantly with day length ($F_{4,262} = 20$, $p < 0.001$; Table 1). The relationship with body mass was negative (slope -0.00011 g^{-1}, $p < 0.01$), indicating a decrease in RQ when birds became heavier. No interaction term contributed significantly to the explained variance. Because no effect of strain on 24-h RQ was detected, we compared the mean 24-h RQ between conditions irrespective of strain (Fig. 6A).

The RQ at the end of the night, after correction for body mass, varied significantly with day length ($F_{4,243} = 5.4$, $p < 0.001$): RQ increased with longer day lengths (Table 1, Fig. 6A). Day length × body mass interaction significantly increased the explained variance ($F_{4,243} = 15$, $p < 0.001$), due to a significant positive relationship between RQ and body mass in both the 9LF ($F_{1,17} = 4.9$, $p < 0.05$) and 18LF group ($F_{1,80} = 33$, $p < 0.001$). Neither strain nor other interaction terms significantly increased the explained variance.

For a better insight in how the variations in metabolic rate and RQ as a function of day length are generated, we plotted for the two most extreme day lengths (6 and 18 h) the 24-h rhythms in metabolic rate (O_2 consumption, VO_2; $L \cdot h^{-1}$) and RQ (Fig. 7). For this we used for both day lengths the data of five broilers of around 100 g (6LF: 87 to 129 g, 18LF: 89 to 112 g). This weight is reached when rates of body weight gain

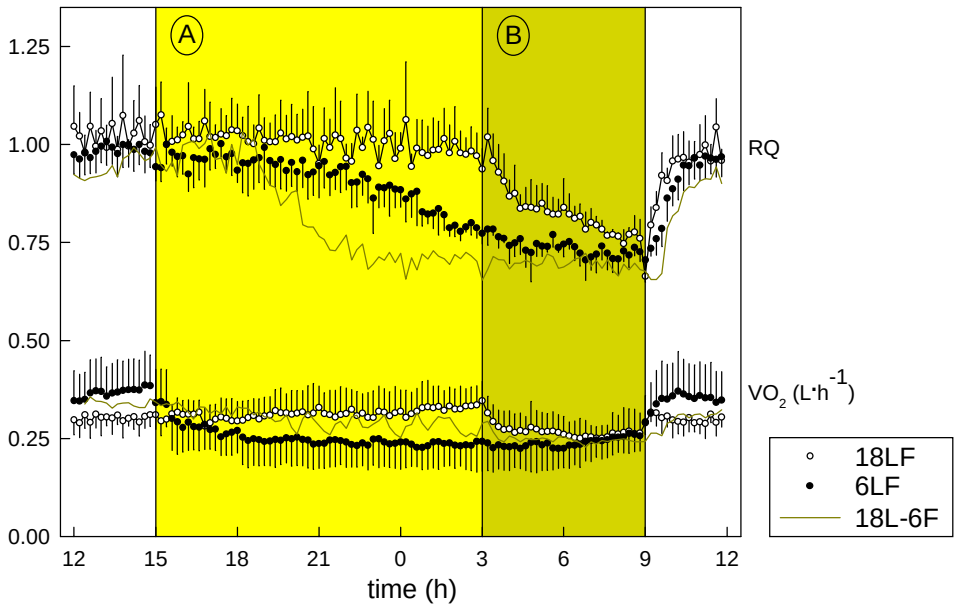


Figure 7. The 24-h rhythms in O_2 consumption (VO_2; $L \cdot h^{-1}$) and RQ for Japanese quail subjected to an 18-h (18LF) or 6-h day length (6LF) with *ad lib.* food during the whole light period, or to an 18-h day length with *ad lib.* access to food during the first 6 h of the light period (18L-6F). (A) Lights-off for the 6LF group and start fasting period for both the 6LF and 18L-6F group. (B) Lights-off for both the 18LF and 18L-6F group and start fasting period for the 18LF group. Values are means, and bars indicate SDs.

are highest (Fig. 1A, 3A), and therefore the largest effects of day length can be expected. In both groups O_2 consumption levels were highly synchronised to the light–dark schedule: levels were high during the light period and decreased after lights-off (Fig. 7). Chicks of the 6LF group showed a tendency to higher O_2 levels during the day than birds subjected to an 18-h day length. On the other hand, O_2 levels during the night in the 6LF group were biased to lower values than in the 18LF group. The 6LF group showed a rise in O_2 consumption about 2 h prior to lights-on, which was not shown by the 18LF group (Fig. 7). RQ was also highly synchronised to the light schedule with high values during the light phase and lower values during the dark phase (Fig. 7). The decrease in RQ after lights-off was quick in the 18-h day length group, whereas chicks in the 6LF group were able to maintain a rather high RQ during the first part of the night. The RQ started to decline about 6 h after lights-off (Fig. 7). The RQ reached its lowest level after about 12 h darkness in the 6LF group, while birds of the 18LF group had not reached that level by the end of the night.

Sexual maturity

Reaching sexual maturity was dependent on experimental condition in both strains (Table 2). Age and body mass at which the first egg was laid and egg mass of all eggs

Table 2. Number of birds laying at 71 d of age, total number of eggs laid during the experimental period, and age (d) and body mass (g; mean ± SD) at the first egg and egg mass (g; mean ± SD) in two strains of Japanese quail subjected to different day lengths and food duration.

treatment	laying animals (*n*)	eggs (*n*)	age (d)	body mass (g)	egg mass (g)
Exp. 1					
broilers					
18LF[†]	4 (5)[‡]	66	52 ± 10	334 ± 20	11.8 ± 0.9 (62)[¶]
15LF	6 (7)	110	45 ± 3	318 ± 25	12.1 ± 1.1 (102)
12LF	1 (5)	1	71	330	13.6 (1)
9LF	0 (6)	-	-	-	-
6LF	0 (2)	-	-	-	-
layers					
18LF	4 (4)	93	43 ± 3[a§]	229 ± 24[a]	11.0 ± 1.4 (92)[a]
15LF	5 (5)	115	43 ± 5[a]	214 ± 9[a]	11.5 ± 2.0 (103)[a]
12LF	2 (3)	38	48 ± 1[a]	226 ± 14[a]	11.3 ± 0.9 (38)[a]
9LF	0 (3)	-	-	-	-
6LF	0 (8)	-	-	-	-
Exp. 2					
broilers					
15L-9F	1 (5)	15	56	401	14.6 ± 0.9 (14)
18L-6F	1 (1)	2	69	241	10.4 ± 0.4 (2)
layers					
15L-9F	7 (8)	127	48 ± 2[af]	194 ± 25[a]	11.3 ± 8.7 (110)[a]
18L-6F	7 (11)	84	56 ± 8[b]	166 ± 11[b]	9.4 ± 0.9 (78)[b]

[†] For definitions, see methods section.
[‡] Numbers in brackets indicate total amount of females per group.
[¶] Numbers in brackets indicate total amount of unbroken eggs.
[§] Numbers with the same letter within experiment 1 are not significantly (Tukey, $p > 0.05$) different from each other.
[f] Numbers of the 15L-9F and 18L-6F group with the same letter as the groups with the same day length in experiment 1 are not significantly (*t* test, $p > 0.05$) different from each other.

laid during the experiment were only tested for the layers: the numbers were too small for the broilers (Table 2).

Experiment 2: food duration

To test if the effect of day length on body mass gain, as found in experiment 1, was mediated solely by the effect of day length on food availability we conducted a second experiment. In this experiment we exposed chicks to long day lengths (15 or 18 h) with *ad lib.* food during a limited part of the light period (first 9 or 6 h, respectively): 15L-9F and 18L-6F, respectively. In his way we could compare these groups with the groups of experiment 1 that were either exposed to the same day length (15LF and 18LF) or the same food availability (9LF and 6LF).

Weight gain

In figure 1 we included with the results of experiment 1 body mass against age for chicks subjected to limited food availability during a long day length. The pattern of weight gain of these chicks showed the greatest resemblance to that of birds with the same food availability: both groups ceased to grow after food restriction was introduced at 7 d of age and increased weight again from 9 d of age onwards (Fig. 1). This effect was clearest in the 18L-6F group, as in the 6LF group. Comparing the total body mass increase, irrespective of sex, with the groups subjected to either the same day length or food availability revealed that weight increase in both strains of the 18L-6F group was significantly lower than the increase in chicks with the same day length (18LF), and did not differ from birds with the same food availability (6LF; Table 1, Fig. 2). In the 15L-9F group weight increase in both strains could not be distinguished from the increase in either the group with the same day length (15LF) or food availability (9LF; Table 1, Fig. 2). The pattern of weight gain rate of the groups with limited access to food during a long day length was again more like the groups with the same food duration than like those with the same day length (Fig. 3).

Gross energy intake

GEI for the groups with limited access to food during a long day length is plotted as a function of body mass at the right side of figure 4. As in experiment 1 we fitted biphasic regression models of log GEI with log body mass. Only in the 15L-9F group a biphasic allometric regression model fitted the data significantly better than a linear regression model (broilers: $F_{2,33} = 4.8$, $p < 0.05$; layers: $F_{2.44} = 3.4$, $p < 0.05$; Fig. 4).

GEI, after correction for body mass, varied significantly with food duration when comparing chicks with restricted food availability during a long day length with birds of experiment 1 that were subjected to the same day length (15-h day length: $F_{1,185} = 13$, $p < 0.001$; 18-h day length: $F_{1,159} = 9.0$, $p < 0.005$): intakes were higher in birds exposed to longer feeding hours. Only treatment × body mass interaction significantly increased the explained variance when comparing the two 18-h day length groups ($F_{1,159} = 8.3$, $p < 0.005$): slope between GEI and body mass was steeper for the 18L-6F group (Fig. 4). Because this interaction was not significant when comparing the two 15-h day length groups, we assumed parallel slopes (0.65) for the individual regressions of GEI against body mass. The resulting intercepts indicated that the GEI level for 15LF chicks was 9.8% higher than for 15L-9F chicks of equal body mass. For none of the comparisons did strain increase the explained variance after correction for body mass.

Comparing GEI between the groups of both experiments with the same food availability, ANCOVA only revealed a significant day length effect, after correction for body mass, when comparing the groups with a daily 6-h feeding period ($F_{1,133} = 6.8$, $p < 0.025$). None of the possible interaction terms significantly increased the explained variance. We therefore assumed parallel slopes (0.72) for the individual regression of GEI against body mass, and calculated that the GEI level for the 6LF chicks was 16% reduced compared to 18L-6F chicks of equal body mass. No difference in GEI between the two strains after correction for body mass was detected.

We plotted the food intake rate (for calculation see results experiment 1) for both groups in the right panel of figure 5. In both strains the intake rates were highest in chicks subjected to daily feeding hours of 9 h or less, irrespective of day length. In broilers the intake rate was significantly higher in the 18L-6F group compared to the 6LF group (24%; Fig. 5).

Energy expenditure

For each treatment and strain we fitted linear regression models between metabolic rate and body mass, because there were no indications that a biphasic pattern between metabolic rate and body mass would improve the fits (Fig. 4). For more details, see experiment 1. Comparing EE between the groups of both experiments with the same day length showed that, after correction for body mass, EE varied significantly with food duration (15-h day length: $F_{1,108} = 23$, $p < 0.001$; 18-h day length: $F_{1,117} = 6.3$, $p < 0.025$): EE was higher in the groups with longer feeding hours (Fig. 4). Food duration × body mass interaction significantly increased the explained variance ($F_{1,117} = 4.1$, $p < 0.05$) when comparing the two 18-h day length groups: slope was steeper in the group with limited food availability (18L-6F). This interaction term did not improve the explained variance when comparing the two 15-h day length groups. We therefore assumed parallel slopes (0.67) for the individual regressions of EE against body mass for this comparison. Intercepts showed that the EE level for the 15LF group was 9% higher than the level for 15L-9F chicks of equal body mass. For none of the comparisons did strain or other possible interaction terms significantly increase the explained variance. RMR also varied significantly with food duration after correction for body mass (15-h day length: $F_{1,108} = 46$, $p < 0.001$; 18-h day length: $F_{1,116} = 13.2$, $p < 0.001$): RMR was higher in the groups with longer feeding hours (Fig. 4). Of the other possible effects, strain ($F_{1,116} = 5.1$, $p < 0.05$) and the interaction between treatment and body mass ($F_{1,116} = 870$, $p < 0.01$) significantly improved the explained variance in RMR when comparing the two 18-h day length groups: RMR level was 4% higher for broilers than layers of equal body mass, and slope of RMR against body mass was steeper in the 18L-6F group. Because day length × body mass interaction was not significant when comparing the two 15-h day length groups, we assumed parallel slopes (0.67) for the individual regressions of RMR against body mass. This yielded a 17% higher RMR level for 15LF chicks compared to 15L-9F chicks of equal body mass.

When we compared EE and RMR between the groups of both experiments with the same food availability, ANCOVA only revealed an effect of day length on EE, after correction for body mass, when comparing the two groups with a daily 6-h feeding period ($F_{1,68} = 819$, $p < 0.001$). None of the other effects improved the explained variance in EE. We assumed parallel slopes (0.79) for the individual regressions for EE against body mass, showing that EE level for 6LF chicks was 14% reduced compared to the level for 18L-6F chicks of equal body mass.

Respiratory quotient

Comparing mean 24-h RQ between the groups of both experiments with equal day length revealed that, after correction for body mass, 24-h RQ varied significantly with

food duration (15-h day length: $F_{1,80}=14$, $p<0.001$; 18-h day length: $F_{1,115}=71$, $p<0.001$): RQ was higher in birds with longer feeding hours (Table 1, Fig. 6). There was no difference in mean 24-h RQ when comparing the groups with equal food availability but different day length. For none of the comparisons did strain or the interaction terms significantly increase the explained variance.

The RQ at the end of the night, after correction for body mass, varied significantly with food duration when comparing the groups of both experiments with equal day length (15-h day length: $F_{1,81}=18$, $p<0.001$; 18-h day length: $F_{1,108}=47$, $p<0.01$): RQ was higher in birds exposed to longer feeding hours (Table 1, Fig. 6). The RQ at the end of the night varied significantly with day length when comparing the groups of both experiments with equal food availability (6 h: $F_{1,63}=23$, $p<0.001$; 9 h: $F_{1,59}=8$, $p<0.01$): RQ was higher in birds subjected to longer day lengths (Table 1, Fig. 6). Neither strain nor the interaction terms significantly increased the explained variance in all comparisons.

As in experiment 1, we plotted the 24-h rhythms in O_2 consumption ($L \cdot h^{-1}$) and RQ of the group of experiment 2 with the same day length and food availability as the two groups plotted in figure 7. For this we used the data of one broiler of about 100 g (comparable to experiment 1). O_2 level in this bird was synchronised to the light–dark schedule: levels remained high during the light period even though food was removed at 1500 hours, and levels dropped during the dark period. As in the 6LF group this bird was able to maintain a high RQ level at the beginning of the fasting period. However, already after 4 h there was a pronounced decrease in RQ and the lowest value was reached almost 3 h earlier than in the 6LF group (Fig. 7).

Sexual maturity

The time at which sexual maturity is reached was modulated by food availability (Table 2). Females of the 18L-6F group did reach sexual maturity despite food restriction, but at a later age and at a lower body mass. These birds also showed a tendency to produce smaller eggs. These effects were either absent or less clear in the 15L-9F group (Table 2).

DISCUSSION

This experiment showed that day length, by determining the period in which daily activity and feeding can occur, had major effects on body mass gain in young birds. We demonstrated in experiment 1 that longer day lengths are related with both higher energy intake and energy expenditure levels, resulting in larger weight gains. The day length below which detrimental effects on weight gain were made visible was 9 h for both strains. Possibly, a 9-h day length is just long enough to compensate for a reduction in energy intake by an almost equivalent decrease in energy expenditure, resulting in weight gains comparable to values found at longer day lengths. Experiment 2 showed that this effect of day length on body mass gain is mainly due to the effect of day length on food availability. Weight gain in the group of birds with access to food during 6 h of an 18-h day length showed a pattern that was

comparable to the group with the same food availability (6LF), but not with the same day length (18LF). We also demonstrated that day length had a significant effect on sexual maturation and that food restriction seemed to delay, but not inhibit this process.

Net weight gain is the resultant of a positive balance between energy intake and energy expenditure. By manipulating day length (experiment 1) we influenced both daily energy intake and daily energy expenditure, with consequences for overall weight gain (Fig. 2). A reduction in day length to 9 h or less affected body mass most during the first two days of the experiment (Fig. 1). During this period the birds lost weight or body mass remained the same. After that body mass increased again, and during the third week of the experimental period no difference in average rate of body mass gain was visible between the different day lengths (Fig. 3). This recovery in weight gain was mainly accomplished by adjustments in daily feeding behaviour (Fig. 4, 5). Already on the second day of the experiment birds subjected to a 9-h day length or less started to exploit their crop as a temporary storage place for food. This food was mobilised when no food was present, as was evident from the maintenance of a high RQ during the first part of the night by the 6LF group (Fig. 7). In this way the birds managed to consume more food than would be expected on the basis of time reduction only, and were able to grow continuously after the initial drop in weight gain. They were however unable to increase their food intake to such an extent that they consumed the same amount or more as birds subjected to day lengths of at least 12 h. Crop (and digestive tract) filling is a generally adopted feeding strategy by birds possessing a crop that enables them to tide over prolonged fasting (nocturnal) periods (4,9,24,70,142,180). The effect of day length on food intake found here was opposite to the effect of day length on food intake as found in pigeons that were gradually transferred from a 12-h to a 3-h day length with only *ad lib.* food during the light period: food intake increased when the birds were gradually subjected to shorter day lengths (9). These animals were however full-grown with lower feeding rates than the chicks investigated here. It is therefore possible that these birds did not experience a feeding period of only 3 h as time limited and were able to increase their food intake even during such a short period.

The effect of day length on food intake induced also diurnal fluctuations in metabolic status, as shown by the 24-h rhythms in RQ (Fig. 7). These fluctuations also influence the overall weight gain chicks achieve at different day lengths. The mean 24-h RQ showed that birds subjected to the two longest day lengths (15 and 18 h) were mainly metabolising carbohydrates ($RQ > 0.9$) These chicks maintained an overall positive energy balance, which allowed for continuous body mass increase. However, when day lengths decreased to 12 h or less the mean 24-h RQ dropped below 0.9, indicating that birds relied for a larger part of the 24-h period on body reserves (fat) for energy (Fig. 7). In this postabsorptive state the energy balance becomes negative, resulting in either a cessation of weight gain or weight loss during a (large) part of the night. These birds show a discontinuous growth curve: body weight increase during the day, and part of the night, alternates with a cessation of weight gain or weight loss during the rest of the night. Overall, birds were clearly in in a net positive energy balance as they grew over the experimental period. The amplitude of weight gain and weight loss should become higher when day length

decreases with less overall growth. The mean RQ over the last half hour of the night confirmed this finding. Several studies in rats and birds have shown that the RQ drops dramatically at the end of a fasting period (9,85,145,178). This drop in RQ results from the depletion of nutrients for fuel for energy metabolism, which leads to the catabolism of fat stores (145). Our results showed that a daily feeding period of 12 h or less is too short to enable quail chicks to store enough food in their crop (and digestive tract) to prevent them from reaching a postabsorptive state during the next night. Hummingbirds, with their high metabolic rates, subjected to a 12-h day length also reach RQ values close to 0.70 at the end of the night, despite food storage in their crop (142,190). Pigeons can endure a daily overnight fast of 21 h without reaching a postabsorptive state (9), and the mature turkey requires up to two days to become postabsorptive (93). The young birds investigated here are fast growing animals, indicating that energy demands are likely to be high, as in adult hummingbirds.

The 24-h energy expenditure (EE) and resting metabolic rate (RMR) were both positively correlated with day length: longer day lengths create more possibilities for locomotor and feeding activities, which are the main parameters that determine metabolic rate. RMR was calculated as the lowest value of a 30-min running mean over the last 24 h of the energy measurement. This value is reached during the night in day-active animals, when activity and food intake levels are lowest or absent. Comparing the 24-h O_2 consumption rhythms of the two most extreme day lengths (6 and 18 h; Fig. 7) showed that a short day length was associated with a high-amplitude cycle in metabolic rate: chicks had higher daytime and lower night-time expenditure levels compared to chicks exposed to a long day length, resulting in lower RMR values. Because of the long night, this high-amplitude in metabolic rate also resulted in a lower EE: lower values during a long dark period more than compensated for higher levels during a short light period. Shorter nights, on the other hand, leave less time for the subsidence of after-effects of daytime food intake and activity, resulting in both higher RMR and EE levels. By incorporating the mean body mass for all ages (Fig. 1) into the regression models for both GEI and EE (Fig. 4), we estimated, via extrapolation, GEI and EE at every age (7–71 d) for all day lengths. Subsequently, we added all results together to a total figure for both GEI and EE per day length over the total experimental period (Fig. 8). In broilers the effect of day length on net energy balance is mainly achieved via changes in GEI: the decrease in GEI from an 18 to a 6-h day length was 37%, while EE decreased with 33%. The corresponding values for layers were 22% and 34%, suggesting that birds that are not selected for high levels of food intake influence their net energy balance more by changing their expenditure levels. GEI appeared to reach its highest level at a day length of 15 h in broilers, and 12 h in layers. EE was linearly related to day length in both strains. These variations in GEI and EE as a function of day length suggest that a 15-h day length is most beneficial for body mass gain in broilers, as was implied by the total body mass increase (Fig. 2). In layers it is less clear. The net energy balance seemed most positive at a 12-h day length, while total body mass increase was not highest at this point (Fig. 2).

We showed that young birds subjected to day lengths of 9 h or less develop different strategies to cope with food restriction. They decrease nocturnal metabolic

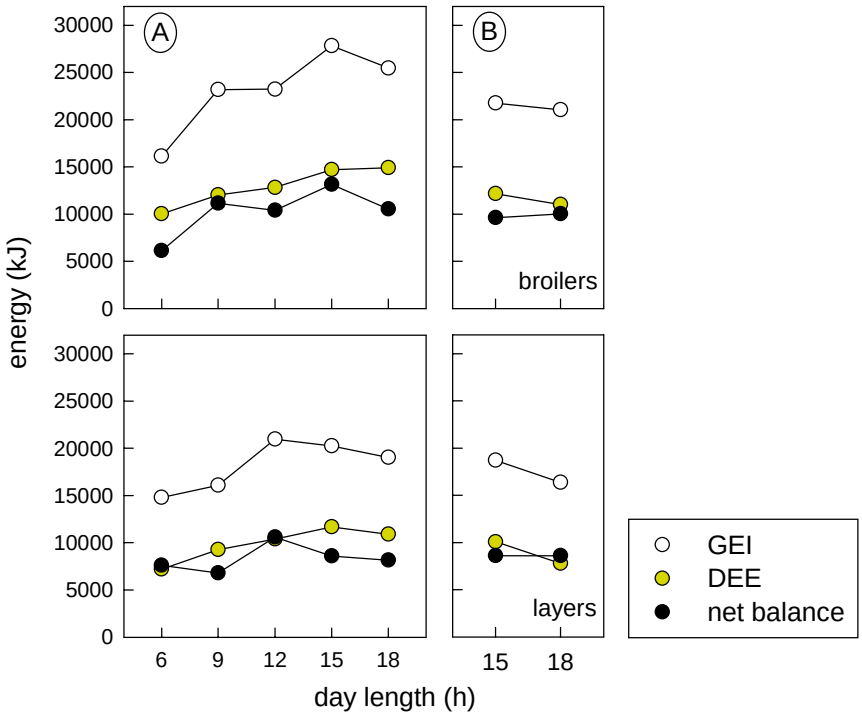


Figure 8. Overall gross energy intake (GEI; kJ), 24-h energy expenditure (EE; kJ) and net balance (kJ) as a function of day length (h) in Japanese quail with *ad lib* food during (A) the whole light period (experiment 1), and (B) first part of a 15-h or 18-h light period (9 or 6 h, respectively; experiment 2).

rates and exploit crop filling to enhance energy intake. Apart from these adaptations, a rise in O_2 consumption prior to lights-on, as shown by the 6LF group (Fig. 7), can also be considered as an adaptive strategy to deal with food restriction. Both mammals and birds when subjected to restricted feeding regimes show increased levels of various response measures as feeding time approaches. Rats for example show increased levels of locomotor activity prior to feeding (114,130), and pigeons are known to show increased levels of O_2 consumption and body temperature (9,145). These increased levels may be an indicator for the activation of several processes in the body in anticipation of food arrival. For example, the anticipatory rise in O_2 consumption and body temperature prior to feeding may, apart from increased locomotor activity, be the result of increased enzyme and/or duodenal activity (34,157). These activities are known to increase in anticipation of food arrival when animals are subjected to periodic food availability (34,157,167). In this way a high efficiency of food digestion is accomplished. Possibly, the increase in metabolic rate prior to feeding in chicks subjected to long fasting periods is an indication of an increased readiness for food intake, resulting in a more efficient use of nutrients. Lower nocturnal energy levels during longer nights (Fig. 7) are possibly associated

with lower body temperatures, as shown in pigeons (9,145). This suggests that metabolic processes are turned down. Entering a new feeding period from a low metabolic position may reduce the efficiency with which birds can utilise consumed food during the following feeding period. To make optimal use of the feeding period the birds may raise metabolic processes preceding food access. We did not distinguish if this anticipatory rise in O_2 consumption was due to anticipation to light or feeding. However, in pigeons the rise in O_2 consumption also appeared before a meal when this did not coincide with lights-on, indicating that birds are able to anticipate feeding times irrespective of light (145).

Day length further had a major effect on sexual development: chicks subjected to 9-h day lengths or less did not reach sexual maturity at 71 d of age. In quail sexual development is known to depend on the length of the daily light period (21,48,78,131,176): long day lengths, from 12 h onwards (48), stimulate sexual maturation, while short day lengths inhibit (21,48,78,131) or delay sexual maturation (176), depending on how long birds were followed. We found that short day lengths inhibited sexual maturation. Female Japanese quail do reach sexual maturation when exposed to day lengths of 6 h, but only at ages over 115 d (176). Therefore, day length may have delayed rather than prevented sexual development. Sexual maturity also influenced body mass gain in female chicks. Females that did not reach sexual maturity remained as small as males.

Broilers grew faster than layers, irrespective of experimental condition (Fig. 2, 3). We expected that broilers, selected for high food intake, would be more sensitive to reductions in day length, and consequently food availability, than layers. There was no evidence for a difference in sensitivity, as day length × strain interaction did not contribute to the explained variance in weight gain (Fig. 2). Broilers had higher GEI levels, even after correction for body mass. Since EE after correction for body mass did not differ between the strains, the higher GEI explains the higher rate of weight increase in broilers.

Experiment 1 showed that day length had a major effect on body mass gain by influencing both energy intake and energy expenditure. To separate the effect of day length from that of food duration we conducted experiment 2. This experiment showed that the effect of day length on overall weight gain was mediated by the effect of day length on food availability: overall weight gain in the 18L-6F group was equal to that of the group with the same food availability (6LF), and lower than that of the group with the same day length (18LF) in experiment 1. Also the mean 24-h RQ seemed to indicate that the effect of day length on body mass gain was realised via food availability: the overall 24-h digestive activity of chicks in the 18L-6F group did not differ from that of the 6LF birds. The way in which the same overall weight gain was achieved in the two groups with a daily 6-h feeding period showed that day length did have an effect on energy intake and energy expenditure independent of food availability. Day length, irrespective of food duration, induced both higher energy intake and energy expenditure levels, resulting in similar overall weight gains. The higher EE as a consequence of 12 h extra light without food may have induced higher energy intake levels by increasing energy requirements. During this time the chicks were restless, possibly searching for food. The rapid drop in RQ in the 18L-6F

group compared to the 6LF group after food removal was indicative for this (Fig. 7). These effects of day length on GEI and EE were not clear when comparing the 15L-9F group with the group of experiment 1 with the same food availability (9LF). Birds subjected to a 9-h feeding period showed a normal weight increase curve (Fig. 1). Interestingly, exposure to a long day length (18 h) allowed birds to reach sexual maturity at lower body masses, despite restricted feeding regimes. The short-day length response in sexual maturation is not mediated by the food restriction imposed by day length. After maturation the birds laid slightly smaller eggs, associated with their reduced body mass (Table 2).

In conclusion, the experiment showed that changes in feeding behaviour and energy expenditure, in response to day length and food availability manipulations, are part of a general strategy in chicks to maximise energy intake and minimise energy expenditure that enables them to gain weight continuously. When day length, and consequently the feeding period, decreased to 9 h the reduction in energy intake could be compensated by a reduction in energy expenditure. When day length was reduced further, the fasting period became too long to compensate the decrease in energy intake by a beneficial effect of a shorter day length on energy expenditure. These birds therefore showed retardation in weight gain compared to quail exposed to longer day lengths.

CHAPTER 4

EFFECT OF SHORT DAILY FEEDING PERIODS ON BODY COMPOSITION IN JAPANESE QUAIL (*COTURNIX C. JAPONICA*) CHICKS

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Submitted

ABSTRACT

Effect of short daily feeding periods on body mass, body composition, and food intake were investigated in fast growing Japanese quail (*Coturnix c. japonica*). Birds were subjected from 7 till 28 d of age to either a long, 18L:6D (LD; $n = 19$), or short day length, 6L:18D (SD; $n = 22$), with *ad lib.* food during the light period. We examined the effect on lean masses of heart, pectoralis muscle, liver, crop, proventriculus plus gizzard, intestine, spleen, and total dry, fat, and lean mass. Birds were sacrificed either at the start or end of the light period to evaluate the amount of food present in the different segments of the gastrointestinal tract (GIT). Results revealed that LD-birds had a higher body mass, and higher relative dry and fat, and lower relative lean mass than SD-birds. Food intake was reduced in the SD-group, but increased more rapidly during the experiment than in the LD-group. Food restriction resulted in a higher relative crop mass, and a lower relative heart mass at 28 d of age. Intestine length was not affected, while intestine density was higher in the LD-group. SD-quail did not adjust their digestive organs to a lower food intake. This resulted in an over-capacity of the GIT at the start of the experiment, which was gradually reduced due to increasing food intake levels. We conclude that fast growing quail subjected to time-limited food restriction enlarge their relative crop mass, by which they are able to increase food intake to a level which makes morphological adjustments of other segments of the GIT and liver not beneficial.

INTRODUCTION

Restricted feeding conditions, where time during which feeding can occur is limited (for example during meal feeding), lead to behavioural adjustments in both feeding and activity levels: food intake rates are increased, and activity levels are reduced when no food is present (3,17,102). In this way young birds are able to grow at maximum rate, despite limitations in food supply. Apart from behavioural adjustments, restricted feeding regimes may also lead to morphological adaptations of the digestive organs (4,74,140). Meal feeding in young chickens resulted in increased relative weights (expressed as percentage of body mass) of crop and gizzard (4). Intermittent feeding, where chicks were deprived of food on alternate days, led to an increase in the relative weights of liver, pancreas, and gastrointestinal tract, including the small intestine (140). Restricting food intake by limiting the amount of food offered without a time limit, results also in changes in the digestive organs compared to *ad lib.* conditions (134,135,179,200). Food restriction (30% of *ad lib.*) in broiler chickens from 7 to 14 d of age resulted in an increased relative gizzard mass at 14 d of age (134). It has been hypothesised that young birds facing an environmental challenge, such as a reduction in food abundance, may exhibit compensatory changes in rates of metabolism and/or maturation (163). In this way chicks would be able to grow at maximum rate. Extending this hypothesis to include the gastrointestinal tract would imply that by morphological adjustments of the digestive organs chicks may increase feeding efficiency and/or food storage capacity, and thus ensure maximal growth rates. These adaptations of the digestive system are likely to be expensive in terms of energy, and may result in a situation where less energy can be allocated to the development of other organs and/or tissues that are less important for growth. For example, food restriction impairs muscle development in growing animals (46,147,197). Also immune function can be negatively influenced by food restriction (12,62,164).

The present study was conducted to investigate the effect of time-limited food restriction on body composition of fast growing birds during early development. For this purpose we investigated changes in body composition in growing Japanese quail (*Coturnix c. japonica*) exposed to either a long (18 h) or short day length (6 h) with only *ad lib.* food during the light period. We examined the birds during a period of rapid body weight gain (0–28 d of age), because we expected the greatest effects during this period. Japanese quail were used, a species with the fastest growth rate in the family Phasianidae (151), and likely to be responsive to variations in food availability. We examined the development of the heart, pectoralis muscle (minor plus major), liver, different components of the gastrointestinal tract (including crop, proventriculus plus gizzard, and intestine), and spleen. We also measured food intake. To evaluate the amount of food present in the different segments of the gastrointestinal tract, we sacrificed birds either at the start (fasted) or end of the daily feeding period (fed).

METHODS

Animals and housing

Japanese quail (*Coturnix c. japonica*) neonates, of a strain selected for maximum body mass at the age of 5 weeks, were obtained from a commercial quail farm (N.V. Nouwen, Lommel, Belgium). Until the age of 6 d birds were kept in wooden cages (*l*×*b*×*h*: 67×39×44 cm^3) with sawdust bedding in continuous light and *ad lib.* access to quail starter food and water, to ensure maximum possible body mass gain. A 40 W heating lamp was placed in each cage to provide a temperature gradient sufficient for selection of the preferred temperature by the chicks. At 6 d of age the birds were assigned to the experimental conditions in such a way that the average body mass did not differ between the groups. The birds were allocated to either a long, 18L:6D (LD; $n = 19$), or short day length, 6L:18D (SD; $n = 22$; lights on at 0900 hours (MET)), with only *ad lib.* food during the light period. At 6 d of age, the chicks were permitted to habituate to the experimental conditions and allowed to eat *ad lib.* during the whole 24-h period. The experiment started at 7 d of age. Throughout the experimental period a pellet-diet (Institute for Animal Science and Health, ID-DLO, The Netherlands) containing 27.7% (w/w) crude protein and 17 $kJ \cdot wet\ g^{-1}$ (gross energy content as determined by bomb calorimetry; own measurement) was fed. Water was freely available.

During the experiment the animals were housed in wooden cages (*l*×*b*×*h*: 67×39×44 cm^3) with a wire bottom and a 40 W heating lamp. The heating lamp was gradually raised and finally removed to allow the ambient temperature to decrease to room temperature (~21°C) within 3 weeks of age. One feeder and one water container were mounted on the left and right side of the cages, respectively. The containers were separated from the inside by a partition with openings that allowed birds to feed and drink without competition. Feeders were automatically removed and returned using a clock-controlled compressed air system.

Food intake

Total food intake (g) per day was measured 2–3 times per week by weighing the feeders at the start and end of the light (= feeding) period from 7 d of age onwards. On these days birds were weighed (to 0.1 g) at the start of the light period, and food became available after weighing. Spilled food was carefully collected from all parts of the cage and at the side of the cage where the feeder was located. The 24-h gross energy intake (GEI; $kJ \cdot d^{-1}$) per cage was calculated by transforming 24-h food intake (g) to its energetic equivalent (17 $kJ \cdot g^{-1}$). This value was expressed as GEI per bird.

Body composition

Chicks were sacrificed by cervical dislocation at 0, 6, 9, 14, 21, and 28 d of age. At each age and experimental condition, 4–6 birds were selected for carcass analysis in such a way that the group of selected chicks had the average body mass of the total group. Neonates were sacrificed the moment they arrived at the laboratory, before they had had access to food. Chicks of 6 d of age were killed at 0900 hours (MET) before assignment to the treatments. At the remaining ages, 2–3 animals were

Table 1. Characteristics (mean ± SD) of Japanese quail exposed to different day lengths with *ad lib.* food during the light period.

		carcass analyses					
day length	age (d)	*n*	fasted/fed* (*n*)	wet body mass (g)	tarsus length (mm)	wing length (mm)	GEI ($kJ \cdot d^{-1}$)
'pretreatment'							
24L:0D	0	5	0/5	8 ± 2.0	14 ± 1.5	18 ± 2.9	-
	6	5	0/5	32 ± 6.4	20 ± 2.3	39 ± 2.3	-
'treatment'							
18L:6D	9	5	2/3	48 ± 12	23 ± 1.7	59 ± 4.6	194 ± 21 (4)†
	14	5	2/3	98 ± 15	29 ± 1.6	83 ± 4.9	270 ± 45 (4)
	21	4	2/2	163 ± 9.0	37 ± 1.1	100 ± 3.9	376 ± 47 (4)
	28	5	3/2	205 ± 12	37 ± 2.2	106 ± 5.1	442 ± 90 (3)
6L:18D	9	5	2/3	33 ± 4.5	22 ± 0.9	53 ± 2.7	92 ± 13 (7)
	14	5	2/3	65 ± 7.6	27 ± 1.8	75 ± 2.5	148 ± 26 (7)
	21	6	3/3	115 ± 25	31 ± 1.9	95 ± 5.4	263 ± 77 (7)
	28	6	3/3	158 ± 29	35 ± 1.3	106 ± 0.8	306 ± 31 (3)

* Number of birds sacrificed either at the start (fasted) or end of the light period (fed).
† Numbers in brackets indicate amount of cages.

sacrificed at the start (fasted) and 2–3 at the end of the light period (fed; Table 1). We weighed birds (to 0.1 g) individually before sacrificing them. Birds were stored individually in deflated plastic bags at -20°C till analysis.

We thawed the chicks overnight in bags and weighed (to 0.1 g) them preceding dissection. The difference in body mass at death and after thawing was small (0.6 ± 0.5 g). We therefore used body mass at death in the analyses. Prior to dissection we measured the lengths of the right wing and right tarsus. Heart, right pectoralis muscle (major plus minor), liver, and spleen were excised and weighed immediately on a Mettler analytical balance to the nearest 0.0001 g. Gastrointestinal tract (GIT) was removed, of which we distinguished the following segments: (1) crop (plus oesophagus), (2) proventriculus plus gizzard, and (3) intestine consisting of small plus large intestine and caeca. These segments were weighed before and after removal of the content. Content was removed by washing the tissue under streaming tap water. The tissue was then blotted on a paper towel to remove excess water. The intestine of neonates was too tender for this procedure and was cleaned by pressing it between blotting paper. We calculated the content of the different GIT segments as the difference between wet mass of the segment before and after cleaning. The (unstretched) length of the intestine (without caeca) was measured, as well as the length of the paired caeca. Each organ was trimmed of fat. In order to reduce the variation in the cutting, weighing and washing procedures, actions were carried out by the same persons.

Wet body mass (WBM; g) was calculated as the difference between body mass at death and the wet food content of the GIT. Dry mass (g) of carcass remainder and individual organs was determined by oven drying (Hereaus UT6200, Germany) at

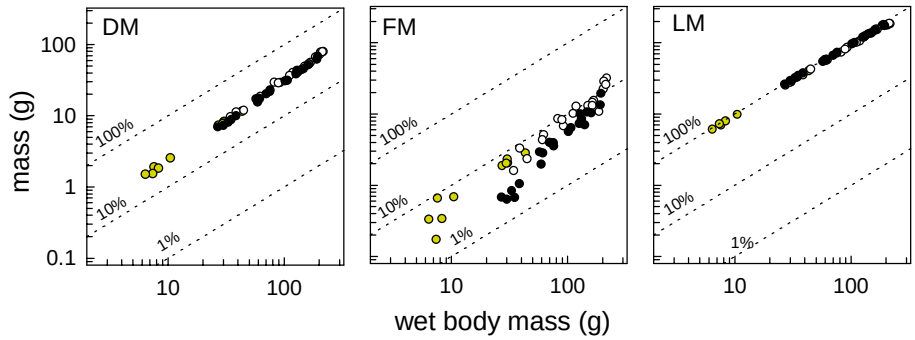

Figure 1. Allometric relationship between total dry (DM; g), fat (FM; g) and lean mass (LM; g), and wet body mass (WBM; g) in Japanese quail subjected either to 24L:0D (grey symbols; at hatching and 6 d of age), 18L:6D (open symbols; at 9, 15, 21, and 28 d of age) or 6L:18D (solid symbols; at ages equal to 18L:6D) with *ad lib.* food during the light period. Diagonal dashed lines indicate percentages of WBM.

103°C for 4–6 h to constant mass (ISO 6496). After weighing the parts, fat was extracted in a Soxhlet apparatus using petroleum-ether (boiling trajectory 40–60°C) as a solvent. After extraction all components were dried for 1 h at 103°C and weighed. We define fat mass (g) of both carcass remainder and each organ as dry mass minus extracted dry mass. Lean mass (g) is defined as the wet mass minus fat mass. Dry, lean and fat organ masses were added to the carcass remainder values to obtain the dry, lean and fat mass of the whole body.

Data analysis

Data are expressed as means and inter-individual standard deviations. Differences between group means were analysed by Student's *t* test (SPSS Inc., 1988). Because of (1) large variations in body mass in the SD-group (Table 1), (2) small sample sizes for each age category, and (3) differences in body mass between the two conditions at the same age, we compared the different parameters based on log-log regressions on body mass from 9 d of age onwards. To this end, we used analysis of covariance (ANCOVA) with experimental condition as main effect and body mass as covariate. No logarithmic transformation was applied when analysing food content of the different gastrointestinal tract segments for a treatment effect with body mass as a covariate. The interaction between treatment and body mass was taken into account to test whether allometric relationships differed between day lengths. If this factor was not statistically significant ($p \geq 0.02$), it was deleted from the model. Subsequently we assumed parallel slopes and calculated the elevations of the regressions of the parameter on body mass for both conditions. A two-tailed significance level of $p < 0.05$ was used in all other tests.

Table 2. Allometric relationship between component mass (g) and body mass (g) in Japanese quail exposed either to a long (18L:6D) or short day length (6L:18D) with *ad lib.* food during the light period.

	exponent ± SE		intercept		R^2	
component	18L:6D	6L:18D	18L:6D	6L:18D	18L:6D	6L:18D
dry mass	1.14 ± 0.019	1.16 ± 0.013	0.1654‡	0.1466	0.995	0.997
lean mass	0.97 ± 0.009	0.97 ± 0.003	1.0517‡	1.0876	0.999	1.000
fat mass	1.32 ± 0.092†	1.67 ± 0.062	0.0190‡	0.0025	0.924	0.973
heart	1.00 ± 0.072†	0.76 ± 0.053	0.0088‡	0.0238	0.920	0.913
pect. muscle	1.29 ± 0.021†	1.40 ± 0.034	0.0254‡	0.0155	0.995	0.988
liver	0.76 ± 0.045	0.92 ± 0.051	0.1196‡	0.0520	0.944	0.941
crop	0.90 ± 0.088	0.99 ± 0.081	0.0097‡	0.0099	0.863	0.883
prov. + gizzard	0.67 ± 0.045	0.73 ± 0.035	0.1261	0.0991	0.927	0.955
intestine	0.73 ± 0.077	0.85 ± 0.048	0.0639	0.0349	0.842	0.940
spleen	0.94 ± 0.156	0.87 ± 0.166	0.0009	0.0014	0.721	0.591

Model was: component mass = aM^b, where M is wet body mass for dry, lean and fat mass, and lean body mass for other components ($n = 19$ for 18L:6D, except for spleen where $n = 16$; $n = 22$ for 6L:18D, except for spleen where $n = 21$). Component mass is expressed as lean wet mass except for the components dry (dry mass of main body), lean (lean mass of main body) and fat mass (fat mass of main body).

† Exponent values for given component differ significantly between day lenghts (*t* test, $p < 0.05$).

‡ Intercept values for given component differ significantly between day lenghts (*t* test, $p < 0.05$).

RESULTS

Body mass, and total dry, fat and lean mass

In table 1 we listed different characteristics of the birds at hatching and 6 d of age (pre-treatment), and 9–28 d of age for both treatments. Chicks subjected to a short day length (SD) did not grow during the first 2 d of the experiment (Table 1). Thereafter wet body mass (WBM; g) increased again, but the chicks did not reach the same WBM at the end of the experiment as the birds exposed to a long day length (LD): WBM was significantly higher in the LD-group ($T_9 = 3.4$, $p < 0.01$). Tarsus (mm) was significantly longer at 28 d of age in the LD-group compared to the SD-group ($T_9 = 2.4$, $p < 0.05$; Table 1). Wing length (mm) at this age was not influenced by treatment (Table 1). Total dry, fat, and lean mass (g) in relation to WBM for both day lengths is plotted in figure 1. Calculated intercepts and exponents for the allometric relationships between component mass and WBM are listed in table 2. Total dry ($F_{1,38} = 23$) and lean mass ($F_{1,37} = 17$) were significantly ($p < 0.001$) influenced by treatment: dry mass was higher (7%) and lean mass lower (3%) in LD-chicks compared to SD-chicks of equal WBM (Fig 1, Table 2). Total fat mass varied also significantly with food duration ($F_{1,37} = 17$, $p < 0.001$) with a significant treatment × WBM interaction ($F_{1,37} = 10$, $p < 0.001$): slope between total fat mass and WBM was steeper in the SD-group (Fig. 1, Table 2). Fat mass in an LD-chick of 30 g (approximate mass at 6 d of age) was on average 2.3 times higher than in an SD-chick

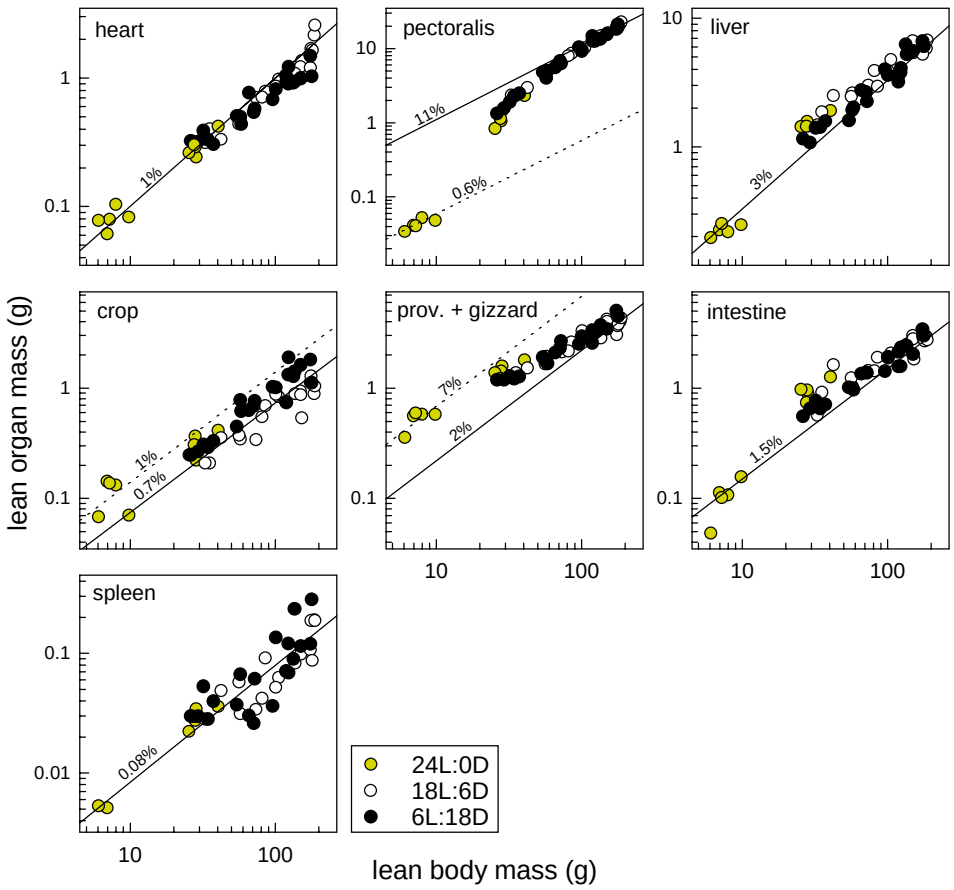

Figure 2. Scaling of different lean organ masses (g) to lean body mass (g) in Japanese quail subjected to three different day lengths with *ad lib.* food during the light period. Diagonal lines indicate percentages of lean body mass (dashed: at hatching; solid: at 28 d of age). For more details, see figure 1.

with equal WBM (approximate mass at 9 d of age). This factor was reduced to 1.2 for chicks of 175 g.

Gross energy intake

Gross energy intake (GEI; $kJ \cdot d^{-1}$) was reduced in the SD-group compared to the LD-group at all ages (Table 1). It is evident that this decrease in GEI was not proportional to the reduction in feeding time. Because almost no food was present in the GIT at the start of the feeding period (Fig. 5A), we assumed body mass at lights-on equal to WBM. Using all GEI data, ANCOVA revealed a significant effect of treatment ($F_{1,76} = 28$, $p < 0.001$) on GEI after correction for WBM, with a significant treatment × WBM interaction ($F_{1,76} = 21$, $p < 0.001$): slope between GEI and WBM was steeper in

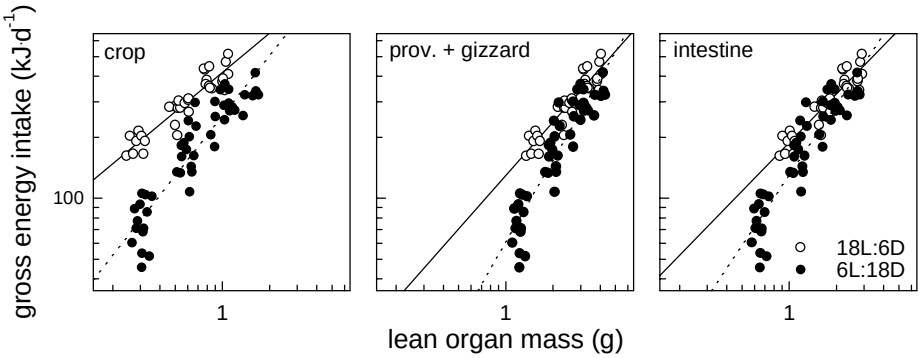

Figure 3. Relationship between gross energy intake (GEI; $kJ \cdot d^{-1}$) and lean organ mass (g) of crop, proventriculus plus gizzard, and intestine in Japanese quail chicks subjected either to a long (18L:6D) or short day length (6L:18D) with *ad lib.* food during the light period. The relationships between GEI and lean organ masses for chicks subjected to a long day length (solid lines) are described by the following equations: $GEI = 418 \times crop^{0.64}$, $GEI = 129 \times (proventiculus\ plus\ gizzard)^{0.88}$, and $GEI = 188 \times intestine^{0.80}$ (for all: $R^2 = 0.929$, $n = 33$, $p < 0.0001$). The equations for chicks subjected to a short day length (dashed lines) are $GEI = 256 \times crop^{0.99}$, $GEI = 61 \times (proventriculus\ plus\ gizzard)^{1.34}$, and $GEI = 1\ 130 \times intestine^{1.15}$ (for all: $R^2 = 0.920$, $n = 47$, $p < 0.0001$).

the SD-group. On the basis of the allometric relationships between GEI and WBM for both a short ($GEI = 3.0 \times WBM^{0.95}$) and long day length ($GEI = 22 \times WBM^{0.57}$), we estimated that an LD-chick of 30 g consumed 77 $kJ \cdot d^{-1}$ (101%) more than an SD-chick of equal WBM. This difference in intake disappeared at a body mass of 175 g: 402 versus 392 $kJ \cdot d^{-1}$. We are aware that some birds contribute multiple points to this analysis. However our design does not permit correction for this.

Organ masses

In figure 2 we compare lean organ masses (g) in relation to lean body mass (LBM; g) between treatments from 9 d of age onwards. Calculated intercepts and exponents for the allometric relationships between lean organ mass and LBM for both treatments are listed in table 2. ANCOVA revealed that lean mass of heart ($F_{1,37} = 5$, $p < 0.025$), pectoralis muscle ($F_{1,37} = 7$, $p < 0.025$), liver ($F_{1,38} = 10$, $p < 0.01$), and crop ($F_{1,38} = 33$, $p < 0.001$) varied significantly with treatment after correction for LBM. Lean liver and crop weighed more (15%) and less (33%), respectively, in LD-chicks compared to SD-chicks of equal mass (Table 2). For both lean heart ($F_{1,37} = 8$, $p < 0.01$) and pectoralis muscle ($F_{1,37} = 7$, $p < 0.025$) treatment × LBM interaction significantly increased the explained variance (Fig. 2, Table 2). None of the other lean organ masses, including intestine, were significantly affected by experimental condition. In figure 2 we plotted diagonal lines that indicate percentages of LBM: dashed lines indicate the percentage at hatching and the solid lines at 28 d of age. The contribution of lean liver, spleen, and intestine mass to LBM was stable throughout development for both conditions (Fig. 2). The contribution of lean pectoralis muscle mass increased largely with

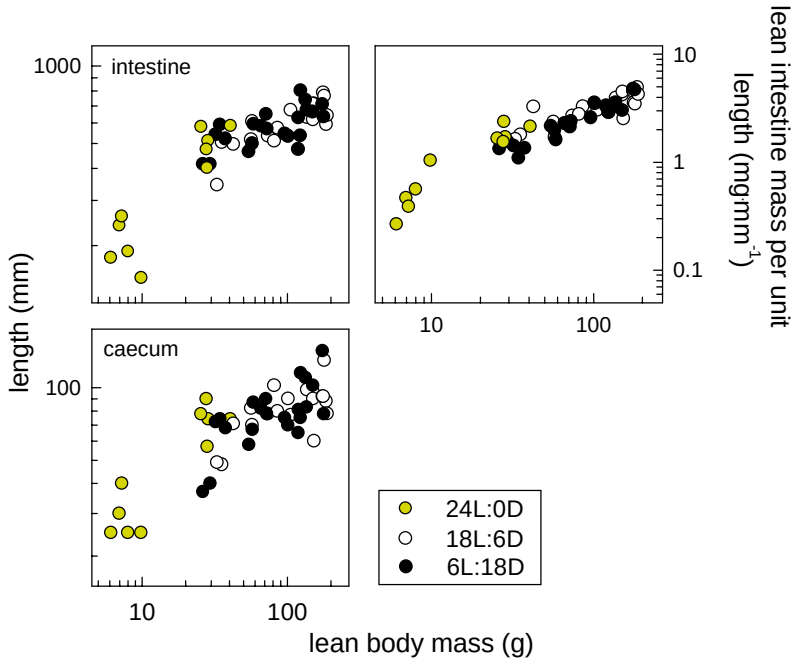

Figure 4. Scaling of intestine length (mm), caecum length (mm), and lean intestine mass per unit length (mg·mm^{-1}) to lean body mass (g) in Japanese quail subjected to three different day lengths with *ad lib.* food during the light period. For more details, see figure 1.

increasing LBM: 0.6 ± 0.1% at hatching versus 11 ± 0.7% at 28 d of age in the LD-group ($T_{4.1}$ = -35, p < 0.001), and 11 ± 1.0% in the SD-group ($T_{5.1}$ = -28, p < 0.001). The percentage of lean crop mass on the other hand decreased throughout maturation for both treatments: 1.5 ± 0.6% at hatching versus 0.6 ± 0.1% at 28 d of age for the LD-group ($T_{4.2}$ = 3.5, p < 0.025), and 0.9 ± 0.2% for the SD-group (T_9 = 2.4, p < 0.05). This was also true for the percentage of lean proventriculus plus gizzard mass: corresponding numbers were 7.0 ± 1.1%, 2.1 ± 0.2% ($T_{4.3}$ = 9.7, p < 0.001), and 2.5 ± 0.2% ($T_{4.3}$ = 8.8, p < 0.0025), respectively. In SD-chicks the contribution of lean heart mass to LBM decreased with time (1.1 ± 0.2% at hatching versus 0.7 ± 0.1% at 28 d of age; T_9 = 3.4, p < 0.01), while this percentage did not change in the LD-group (1.0 ± 0.3%).

GEI in relation to lean mass of the three GIT segments for both conditions is plotted in figure 3. We estimated the lean mass of the different components for a certain GEI by using the equations listed in table 2. For this we converted body mass at the start of the feeding period to LBM, and estimated the lean mass of the three GIT components for a certain LBM (and thus for a certain GEI). Figure 3 shows that shortly after the start of the experiment SD-chicks had not adjusted their GIT organ masses to the lower food level: GEI in these birds was decreased for all three segments

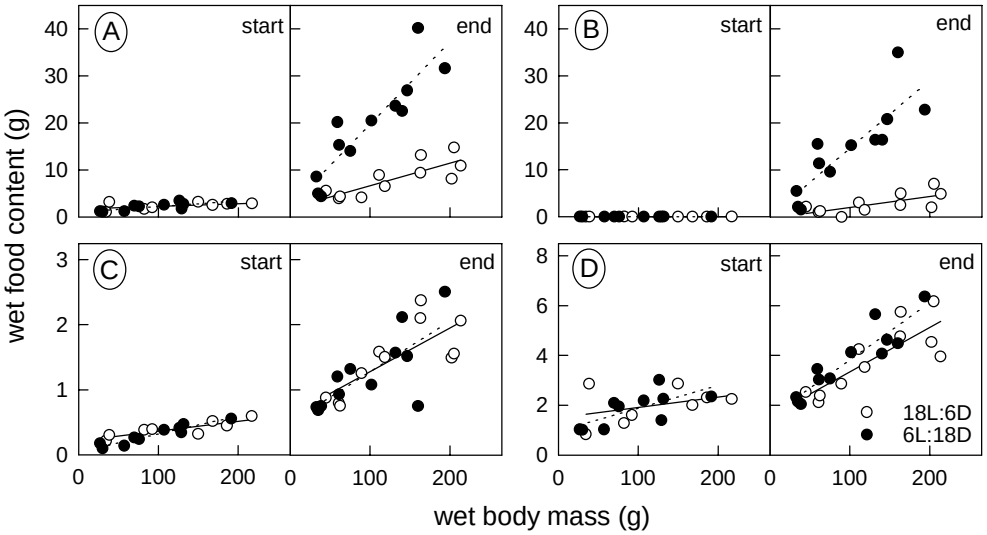

Figure 5. Wet food content (g) of the total gastrointestinal tract (A), crop (B), proventriculus plus gizzard (C), and intestine (D) as a function of wet body mass (g) in Japanese quail subjected to a long (18L:6D; solid lines) or short day length (6L:18D; dashed lines) with *ad lib.* food during the light period. Wet food content was measured either at the start (fasted) or end of the light period (fed).

compared to LD-chicks with equal lean organ mass. Using the allometric relationships between GEI and the different lean organ masses for both treatments (Fig. 3), we calculated that, on average, GEI of an LD-chick with a lean crop mass of 0.3 g (mass at the start of the experiment) was 2.5 times higher than of an SD-chick with equal crop mass. The factors of birds with a lean proventriculus plus gizzard mass of 1.7 g and intestine mass of 1 g (representative masses at the start of the experiment) were 1.7 and 1.4, respectively. Thereafter GEI increased, and at the end of the experiment GEI of SD-chicks was comparable to that of LD-chicks with the same lean mass for both proventriculus plus gizzard (3 g), 391 $kJ \cdot d^{-1}$ versus 437 $kJ \cdot d^{-1}$, and intestine (4 g), 460 $kJ \cdot d^{-1}$ versus 453 $kJ \cdot d^{-1}$. In contrast, SD-chicks were unable to increase GEI to such a level that they approached the intake of LD-chicks with equal lean crop mass: GEI of LD-chicks with a 1 g lean crop mass was on average 1.6 times higher than of SD-chicks with equal lean crop mass. SD-chicks were only able to approach that intake level by enlarging crop mass (Fig. 3).

Figure 4 compares LD- and SD-quail in relation to LBM with respect to intestine length (mm), lean intestine mass per unit length ($mg \cdot mm^{-1}$), and caecum length (mm). Lean intestine mass per unit length varied significantly with day length ($F_{1,38} = 4$, $p < 0.05$): LD-quail had a higher (12%) intestine density than SD-quail of equal LBM. Both intestine and caecum length were not affected by treatment (Fig. 4).

Table 3. Overall wet food content (g; mean ± SD) of the gastrointestinal tract (GIT) and its different segments either at the start (fasted) or end of the light period (fed) in Japanese quail exposed to a long (18L:6D) or short day length (6L:18D) with *ad lib.* food during the light period.

day length	GIT (g)	crop (g)	prov.† + gizzard (g)	intestine‡ (g)
18L:6D				
fasted	2.4 ± 0.8	0.0 ± 0.0	0.4 ± 0.1	2.0 ± 0.7
	***¶	***	***	**
fed	8.1 ± 3.7	2.7 ± 2.0	1.5 ± 0.5	3.9 ± 1.4
6L:18D				
fasted	2.1 ± 0.8	0.0 ± 0.0	0.3 ± 0.1	1.8 ± 0.7
	***	***	***	**
fed	19 ± 11**§	14 ± 9.4**	1.3 ± 0.6	3.8 ± 1.4

† prov. = proventriculus.
‡ intestine = small plus large intestine, and caeca.
¶ Difference between the fasted and fed state within day length (*t* test).
§ Difference between day length within feeding state (*t* test).
*** $p<0.001$, ** $p<0.01$.

Food content gastrointestinal tract

At lights-on the amount of food (g) present in the three GIT segments did not differ between treatments (Fig. 5A). Feeding resulted in a large increase in food content of the total GIT in SD-chicks and to a lesser extent in LD-chicks (Table 3). This increase was largely due to the storage of food in the crop and to a lesser extent in the proventriculus plus gizzard and intestine (Fig. 5, Table 3). The variation in food storage in the crop at the end of the light period was completely explained by WBM ($F_{1,19}=33$, $p<0.001$) and treatment × WBM interaction ($F_{1,19}=17$, $p<0.0025$). Food content of the proventriculus plus gizzard and intestine at the end of the feeding period was not affected by treatment or treatment × WBM interaction after correction for WBM (Fig. 5, Table 3).

DISCUSSION

This experiment showed that young fast growing quail increase their hoarding capacities (by enlarging crop mass) when confronted with time-limited food restriction imposed via day length reduction. In this way chicks are able to increase food intake to a level that enables them (1) to make optimal use of the gastrointestinal tract capacity by the end of the experiment, and (2) to gain weight continuously after an initial drop in body mass at the start of the experiment. No adjustments in proventriculus plus gizzard and intestine were visible.

Gross energy intake (GEI) in growing Japanese quail subjected to daily 6-h feeding periods was reduced at the start of the experiment, but increased more rapidly during the experimental period than in chicks subjected to daily 18-h feeding periods. This

increase in GEI was mainly due to an increase in relative crop mass by which the young birds enlarged their food storage capacities (Fig. 3). This was evident from the large amount of food stored in the crop by the end of the feeding period in SD-birds (Fig. 5, Table 3). Food storage in proventriculus plus gizzard and intestine was minimal compared to crop storage in both treatments (Fig. 5, Table 3). Crop filling is a generally adopted feeding strategy by birds possessing a crop that are subjected to daily periods during which no feeding can occur (4,9,102). By crop filling these birds ensure themselves of a constant flow of energy during (part of) a fasting period. Following this strategy the young quail in this study were able to gain weight continuously after an initial drop in body mass during the first 3 d of the experiment (Table 1). In this way they also managed to normalise relative fat, pectoralis muscle, and liver masses by the end of the experiment (Fig. 1, 2).

A strategy growing animals can adopt to maintain high rates of body weight gain despite lower food intake levels is to allocate energy in preference of certain organs essential for growth (for example digestive organs) at the expense of other, less important organs (for example muscle, spleen). In birds with a high growth rate the growth pattern is characterised by a rapid early development of the digestive organs and a late development of pectoral muscles (89,154). We showed that this pattern of development was hardly affected by food restriction. Although relative pectoralis muscle, heart, and liver mass were decreased in SD-quail at the beginning of the experiment, only the relative heart mass was still significantly reduced by the end of the experiment compared to LD-chicks. Also the immune function, with spleen mass as an indicator, was not affected by food restriction. Spleen is one of the lymphoid organs involved in the defence of the body against antigens, and reduced spleen weights are generally accepted as an indicator for an impaired immune function (11,82,91,92,99). We only investigated the effect on spleen mass and can therefore not exclude that food restriction may have impaired other indicators of the immune system. Thus, in growing quail time-limited food restriction did not lead to an allocation of energy in benefit of certain organs, but resulted in a general retardation of growth of all body tissues relative to body mass.

We had no indications that the storage capacities of the proventriculus plus gizzard and intestine were influenced by food restriction. Nor that food restriction resulted in increased feeding efficiencies: both intestine weight and length were unaffected, while intestine density ($mg \cdot mm^{-1}$) was even decreased in restricted chicks. The digestive organs, especially the small intestine, are generally known for their considerable flexibility when confronted with changing (feeding) conditions, such as changes in energy demands (35,58-60,138), diet (23,73,139), or food availability (4,74,126,140). Lactating rats that face higher energy demands show, besides an increase in food intake, an increase in small intestine length and in masses of stomach, small intestine, and caecum (35). Migratory birds on the other hand, that also face long periods of high energy demand during flight, have reduced masses of gizzard and intestine before migration that are quickly rebuild upon arrival at the migratory destination (138). In this way these birds dispense with costly organs not directly necessary during flight. High flexibility of the small intestine is also visible in broiler chickens that are refed after a short period of early-life food restriction (± 1

week): small intestine increases more rapidly in mass than the whole body (134,135). It was inferred (80) from force-feeding experiments in chicks that digestive organs usually function at maximum rate, and a substantial increase in ingestion can only be accomplished by enlargement of the digestive tract.

The quail chicks in our study did not adjust GIT organs (apart from crop) to a lower food intake level. We showed that at the start of the experiment SD-chicks were unable to make optimal use of their GIT capacity (Fig. 5). Because the GIT, and especially the small intestine, is a metabolically expensive system to maintain in comparison to other body tissues (191), adjusting its size to a lower intake level would be an obvious strategy to follow. Reducing the GIT would enable the birds to economise on total 24-h energy expenditure, leaving more energy for growth. We demonstrated however that, by enlarging the crop, young quail were able to increase food intake to such a level that the utilisation of the capacity of both proventriculus plus gizzard and intestine gradually improved (Fig. 5). This likely made morphological adjustments of these organs even disadvantageous. Adjustments are also likely to be expensive in terms of energy and increasing the storage capacity of the crop (a metabolically inactive tissue) may be an energetically cheap solution to grow as fast as possible. Also, a large GIT at low levels of food intake may enlarge food retention times that could increase digestive efficiency (73). Clearly, more research is warranted on the optimisation of the digestive system.

ACKNOWLEDGEMENTS

The authors thank T. Piersma for his valuable comments.

CHAPTER 5

FEEDING AND BODY MASS OF JAPANESE QUAIL (*COTURNIX C. JAPONICA*) CHICKS WITH UNPREDICTABLE FOOD ACCESS

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Submitted

ABSTRACT

We investigated the effect of unpredictable feeding times on feeding activity and body mass gain in fast growing Japanese quail (*Coturnix c. japonica*) from 7 till 31 d of age. Quail chicks were subjected to a long day length (18L:6D) with *ad lib.* food during (1) the light period, starting 0.5 h after lights-on (group A, $n = 14$), (2) 6 h of the light period, starting 0.5 h after lights-on (group B, $n = 14$), and (3) 6 h of the light period, starting pseudorandomly either 0.5, 6, or 11.5 h after lights-on (group C, $n = 12$). We examined the effects on locomotor, feeding and drinking activity, body mass, and food intake. Treatment did not affect daily locomotor, feeding, and drinking activity. Distribution of activity over the light period was affected: group B and C showed more activity above the feeder and water container outside the feeding time, and showed a large bout of locomotor and feeding activity at the start of this period. Both overall weight gain and gross energy intake (GEI) were highest in group A and lowest in group C. Distribution of food intake throughout the daily feeding period was affected: group B and C consumed more food during the first part of the feeding period than group A. Daily GEI in group C was influenced by fasting duration prior to feeding and by time of food availability. We suggest that young quail adjust their feeding behaviour in response to their instantaneous energy needs. This effect is modulated by time of food availability: food arriving later in the day led to higher intake levels.

INTRODUCTION

Exposure to food restriction, by either reducing the daily amount of food offered or limiting the time during which feeding can occur, is known to have detrimental effects on body mass gain in juvenile birds (3,12,17,52,86,134). When subjected to such a condition, chicks can adopt different strategies to maximise weight gain. They may reduce 24-h energy expenditure by decreasing activity during the time no food is present. In this way more energy will be available for body mass increase. When food restriction is imposed by reducing the time during which food is available, birds can also try to improve weight gain by increasing food intake rates. This may be achieved by enlarging (external or internal) hoarding (7,9,142,196). In most experiments where food restriction is imposed via time limitation, food arrives daily at the same time. From these studies it is difficult to understand what controls feeding motivation during food restriction. Experiments usually combine food restriction with a fixed daily scheduling of food availability, and hence do not distinguish between a number of possible mechanisms that may control food intake rates: (a) the instantaneous energy deficit as built up by a prior fast, (b) the learnt anticipation of the subsequent fasting interval, and/or (c) the circadian time at which food is expected. To start unravelling some of these potential factors in feeding motivation, we studied the effects of daily predictability of feeding in growing birds. Exposing adult birds to unpredictable feeding regimes has shown that they are able to regulate their internal and external energy reserves in response to recent experience or in anticipation of requirements (10,43,72). In Great tits (*Parus major*) body mass increase in response to unpredictable feeding conditions was shown to be an adjustment to the feeding circumstances as experienced in the previous few days (10). In both mammals and birds it is known that the circadian system plays an important role in the anticipation of feeding schedules (20,33,115,130,136). When in rats feeding is restricted to a single meal scheduled at a fixed time, these animals show increased locomotor activity before feeding time (20,67,116,130). This prefeeding activity in rats occurs only when food is offered at intervals near 24 h (1).

In most studies that addressed the effect of unpredictable feeding conditions on food intake and body mass in adult birds, animals were not food restricted: birds were given the choice of either increasing (by enlarging fat reserves) or maintaining their body mass (10,45,56,68,195). It is not known how animals respond to unpredictable feeding regimes when subjected to food restriction. We therefore studied the effect of unpredictable and restricted feeding conditions on body mass and feeding behaviour in growing Japanese quail (*Coturnix c. japonica*). These birds have the fastest growth rates in the family Phasianidae (151), and are likely to be responsive to variations in food availability. In growing animals a substantial amount of the energy intake is needed for growth. It is plausible that unpredictable, restricted feeding conditions during early development may be more critical than in adults with serious consequences for both future reproduction and survival. We subjected the chicks to either of three different feeding regimes: 17.5 h feeding, 6 h feeding with the start of the feeding period at a fixed time every day, and 6 h feeding with the start of the feeding period at an unpredictable time every day. These schedules were chosen to separate the effects of food restriction from those of the predictability of a restricted

schedule. We examined the effects on locomotor, feeding and drinking activity, body mass, food intake, and 24-h distribution of food intake throughout juvenile development.

METHODS

Animals, experimental set up, and housing

Japanese quail (*Coturnix c. japonica*) neonates, of a strain selected for maximum body mass at the age of 5 weeks, were obtained from a commercial quail farm (N.V. Nouwen, Lommel, Belgium). Until the age of 6 d the birds were kept in wooden cages ($l \times b \times h$: $67 \times 39 \times 44$ cm^3) with sawdust bedding in continuous light and *ad lib.* access to quail starter food and water, to ensure maximum possible body mass gain. A 40 W heating lamp was placed in each cage to provide a temperature gradient sufficient for selection of the preferred temperature by the chicks. At 6 d of age the birds were assigned to the experimental conditions in such a way that the average body mass did not differ between the groups. A long day length (18L:6D) was used for all groups throughout the experiment. Group A ($n = 14$) was allowed to eat during 17.5 h of the light period, starting 30 min after lights-on. Group B ($n = 14$) had *ad lib.* food during 6 h of the light period, starting 30 min after lights-on every day. Group C ($n = 12$) had also only *ad lib.* food during 6 h of the light period, but food was offered at three different times on different days: (1) 30 min after lights-on (comparable to group B; Early), 6 h after lights-on (Middle), or (3) 11.5 h after lights-on (Late). In this way also the link between food availability and lights-on was broken. The sequence of the three times was pseudorandomly chosen, so that E, M and L occurred with equal frequency in the experiment, which lasted 24 d (E, M, L, M, L, E, M, L, E, E, M, L, L, E, L, E, M, M, E, L, M, L, M, E). At 6 d of age, the animals were permitted to habituate to the experimental conditions and allowed to eat *ad lib.* during the whole 24-h period. The experiment started at the age of 7 d. Throughout the experimental period a pellet-diet (Institute for Animal Science and Health, ID-DLO, The Netherlands) containing 27.7% (w/w) crude protein and 17 kJ·wet g^{-1} (gross energy content as determined by bomb calorimetry; own measurement) was used. Water was freely available.

During the experiment the animals were housed in wooden cages ($l \times b \times h$: $67 \times 39 \times 44$ cm^3) with a wire bottom and a 40 W heating lamp. The heating lamp was gradually raised and finally removed to allow the ambient temperature to decrease to room temperature ($\sim$21°C) within 3 weeks of age. The birds were housed in pairs except for four cages in group B and two cages in group C with only one bird. This was due to mortality and aggression between birds, and did not affect overall weight gain. One feeder and one water container were mounted on the left and right side of the cages, respectively, and separated from the inside of the cage by a partition containing two openings, one for each bird. Feeders were automatically removed and returned using a clock-controlled compressed air system.

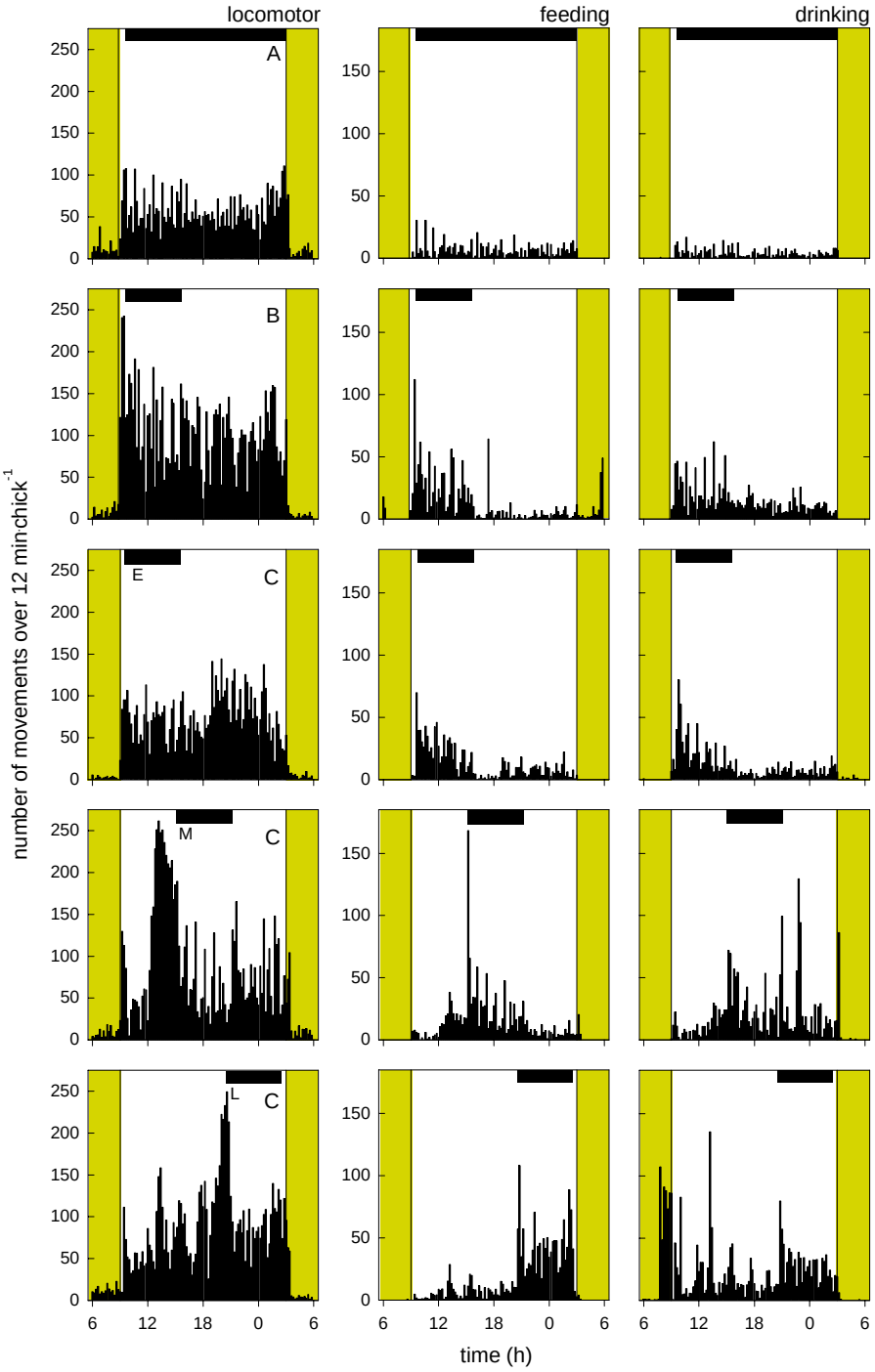
Activity, body mass, and food intake

Locomotor activity was continuously recorded by passive infrared detectors (PID, Wonderex FX-35) placed in the centre above six cages of both group A and B, and eight cages of group C. Feeding and drinking attempts were recorded as interruptions of an infrared beam (XUL-M06031, Telemécanique, France) located above the feeder and the water container. Feeding activity was recorded in three, seven, and six cages of groups A, B, and C, respectively. The number of cages for drinking activity were three, five, and six, respectively. All movements were automatically recorded on a computer every 2 min throughout the experiment and expressed as the number of movements per bird.

Birds were weighed (to 0.1 g) daily before food became available, and every hour afterwards over 6 h to obtain a measure for the distribution of food intake over the feeding period. We are aware that by this procedure birds of the different groups were not proportionally disturbed (group B and C every hour of the feeding period and group A at only one third of that period), which may have affected daily food intake, and consequently body mass. Since weighing took less than a minute per animal, this effect is probably negligible compared to the treatment effect. We chose for weighing the birds hourly instead of the feeders because of food spillage: the time needed to collect spilled food would have interfered with the restricted feeding time. Total daily food intake (g) was measured by weighing the feeders every morning just after lights-on. Spilled food was carefully collected from all parts of the cage and at the side of the cage where the feeder was located. Daily gross energy intake (GEI) was calculated by transforming daily food intake (g) to its energetic equivalent (17 $kJ \cdot g^{-1}$). This value was expressed as GEI per bird. To obtain the daily "growth efficiency" ($\% \cdot d^{-1}$), the change in body mass per 24 h was divided by the amount of food (g) eaten over the same period.

Data analysis

Data are expressed as means and inter-individual standard deviations. Differences between group means were analysed posthoc by Tukey's "honestly significant difference" test, after an effect of treatment on the variable of interest was ascertained by ONEWAY analysis (SPSS Inc., 1988). Student's *t* test was used when comparing only two groups. Analysis of covariance (ANCOVA) was applied, after logarithmic transformation of gross energy intake and body mass, to test for the effect of treatment and treatment × body mass interaction on gross energy intake after correction for body mass. ANCOVA was also used to test for the effect of hours fasting prior to feeding and time of food arrival, and possible interaction terms, on gross energy intake in group C, after correction for body mass. This ANCOVA procedure is an *a posteriori* test without preplanned comparisons and the statistics should be evaluated conservatively (128). Therefore, interaction terms were removed when $p \geq 0.02$. The repeated measures procedure was applied to test for the effect of treatment on the distribution of food intake over the first 6 h of the feeding period. Tests were two-tailed, and significance was accepted at $p < 0.05$.


locomotor
feeding
drinking
A
B
E
C
M
C
L
C
number of movements over 12 min chick-1
time (h)
0
50
100
150
200
250
6
12
18
0
6

RESULTS

Locomotor, feeding, and drinking activity

In figure 1 we plotted representative daily locomotor, feeding and drinking activity patterns for the three groups. Points depict the average activity over all cages during a 24-h period. For group C activity levels were plotted for the three different times of food arrival after an 18-h fast. We chose for an 18-h fast, because of its comparability with the daily fasting duration of group B. Locomotor activity pattern of all groups was highly synchronised with the light–dark cycle (Fig. 1). In all groups more than 90% of the locomotor activity over the whole experiment occurred during the light episode: 91.4 ± 1.8% in group A, 96.9 ± 0.7% in group B, and 96.5 ± 1.0% in group C. About one third of the locomotor activity during the light episode in group B and C occurred during feeding time (equivalent to the duration of the feeding period compared to the light period): 31.4 ± 2.7% and 26.3 ± 5.4%, respectively. The average 24-h locomotor activity, calculated over the total experimental period, was not affected by treatment: 5793 ± 586 movements·d^{-1} for group A, 6441 ± 1887 movements·d^{-1} for group B, and 7917 ± 2070 movements·d^{-1} for group C. The distribution of activity was influenced by treatment (Fig. 1). In group C the postponement of the feeding period to later hours was associated with an irregular locomotor activity pattern, which became more stable at a lower level after food arrival (Fig. 1). There was a rise in locomotor activity in anticipation of food arrival that was absent in group A, and group C on days with early (E) feeding (Fig. 1).

In group A 99.8 ± 0.1% of all activity above the feeder and the water container over the total experimental period occurred during the time of food availability (Fig. 1). In group B and C activity above the feeder was highly correlated with the presence of food, with a peak in activity at the start of food access. In group C this peak showed a tendency to increase when food arrival was postponed to later hours in the day (Fig. 1). After this initial peak, activity above the feeder subsided during the subsequent hours of food access, except in group C at time L: activity remained relatively high and increased at the end of the feeding period (= start of the night). Calculated over the total experimental period 71.5 ± 10.9% of all activity above the feeder in group B and 63.0 ± 12.5% in group C occurred during the period of food availability (Fig. 1). Drinking was less restricted to this period (Fig. 1): in group B 58.8 ± 5.2% of all activity above the water container occurred during food availability, and in group C 45.8 ± 8.7%. Activity above the water container was especially high in group C when food arrived later in the day (Fig. 1). The number of movements above the feeder per 24 h over the total experimental period was not influenced by treatment: 922 ± 280 movements·d^{-1} for group A, 1374 ± 815 movements·d^{-1} for group B, and 1395 ± 329 movements·d^{-1} for group C. This was also true for the total number of

← **Figure 1.** Representative plots of daily locomotor, feeding and drinking activity of Japanese quail chicks subjected to a long day length (18L:6D) with food available during (1) 17.5 h of the light period, starting 0.5 h after lights-on (group A), (2) 6 h of the light period, starting 0.5 h after lights-on (group B), and (3) 6 h of the light period, starting either 0.5 (E), 6 (M), or 11.5 h after lights-on (L; group C).

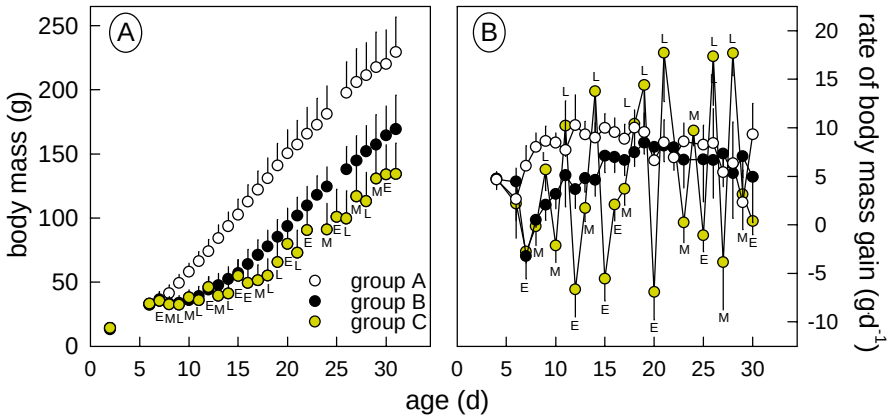


Figure 2. A. Mean body mass (g) and B. mean rate of body mass gain ($g \cdot d^{-1}$) as a function of age (d) in three groups (A, B, and C) of Japanese quail. E, L, and M indicate feeding times in group C. Vertical lines indicate SDs. For more details, see figure 1.

movements above the water per 42 h: 686 ± 39 movements·d^{-1}, 1473 ± 2617 movements·d^{-1}, and 1701 ± 752 movements·d^{-1}, respectively.

Weight gain

Figure 2A compares body mass (g) development against age (d) for the experimental groups. At 7 d of age, when food restriction was introduced, group B and C did not gain weight during the first 2 d. After that weight increased again, but the birds did not reach the same body mass at the end of the experiment as birds of group A. The total body mass increase was highest in group A (194 ± 24 g), and lowest in group C (99 ± 23 g). Group B was intermediate (133 ± 25 g; $F_{2,39} = 54$, $p < 0.0001$).

The rate of body mass gain ($g \cdot d^{-1}$) varied with experimental period (Fig. 2B). Group A showed the highest rate of weight gain during the first 12 d of the experiment. Group B had a low rate of weight gain during the initial part of the experiment, but increased its weight gain rate to the level of group A at 19 d of age. In group C weight gain rate fluctuated: days of rapid weight gain alternated with days of low or no increase in body mass.

Gross energy intake

Mean daily gross energy intake (GEI; $kJ \cdot d^{-1}$) of quail for all treatments is plotted as a function of age in figure 3. Mean daily GEI over the total experimental period was highest in group A (324 ± 32 $kJ \cdot d^{-1}$), and lowest in group C (171 ± 30 $kJ \cdot d^{-1}$). Group B was intermediate (213 ± 19 $kJ \cdot d^{-1}$; $F_{2,22} = 61$, $p < 0.0001$). It is evident from these figures that the decrease in GEI in group B and C was not proportional to the reduction in feeding time. After incorporating mean body mass as a covariate in ANCOVA, the effect of treatment on GEI remained significant ($F_{2,19} = 35$, $p < 0.001$).

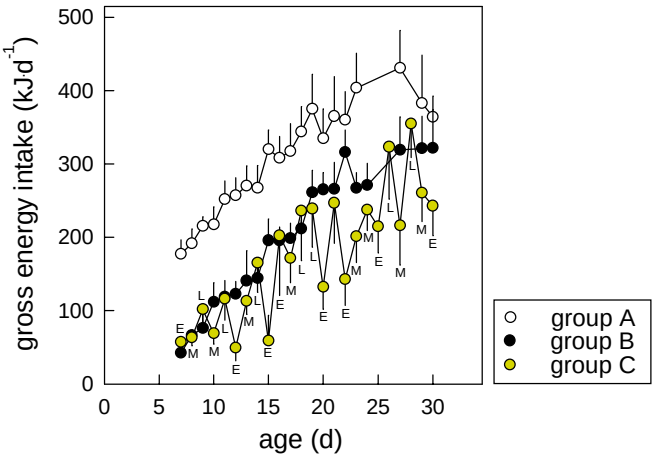


Figure 3. Mean daily gross energy intake (kJ·d^{-1}) in relation to age (d) in three groups of Japanese quail (A, B, and C). E, L, and M indicate feeding times in group C. Vertical lines indicate SDs. For more details, see figure 1.

Treatment × body mass interaction did not significantly contribute to the explained variance.

The difference in mean daily GEI between group B and C, despite equal daily food availability, was due to the variability in both fasting duration prior to feeding and time of food arrival (Fig. 1) in group C. The best way to analyse the effect of these two factors on GEI would be to include both simultaneously in a single ANCOVA, and test the contribution of each to the explained variance. However, by manipulating the sequence of feeding time we could not control simultaneously for fasting duration. Length of fasting was therefore not evenly distributed over the three feeding times, making an analysis with repeated measures not feasible. We therefore analysed the effect of fasting duration on mean GEI corrected for body mass (kJ·d^{-1}·g^{-1}) within group C irrespective of feeding time. ANCOVA revealed that mean GEI varied significantly with fasting duration ($F_{4,35}=86$, $p<0.0001$; Fig. 4). Up to an 18-h fast, an increase in fasting duration was related to an increase in GEI. Fasting for more than 18 h did not increase GEI any further. Daily GEI per g animal (kJ·g^{-1}·d^{-1}) after an 18-h fast was significantly higher in group C than group B, 3.4 ± 0.3 kJ·g^{-1}·d^{-1} and 2.6 ± 0.2 kJ·g^{-1}·d^{-1}, respectively ($T_{14}=$-5.8, $p<0.001$). To examine the effect of feeding time on mean GEI, we calculated the mean daily GEI per g animal (kJ·d^{-1}·g^{-1}) for the three feeding times, irrespective of fasting duration. Time of food arrival significantly influenced daily GEI ($F_{2,21}=158$, $p<0.0001$): GEI was lowest at time E (1.9 ± 0.2 kJ·d^{-1}·g^{-1}), intermediate at time M (2.4 ± 0.1 kJ·d^{-1}·g^{-1}), and highest at time L (3.5 ± 0.2 kJ·d^{-1}·g^{-1}).

The mean daily growth efficiency (%·d^{-1}) was stable throughout the study in group A (Fig. 5). The two restricted groups had a negative growth efficiency at the start of the experiment. In group B growth efficiency increased rapidly and reached the same

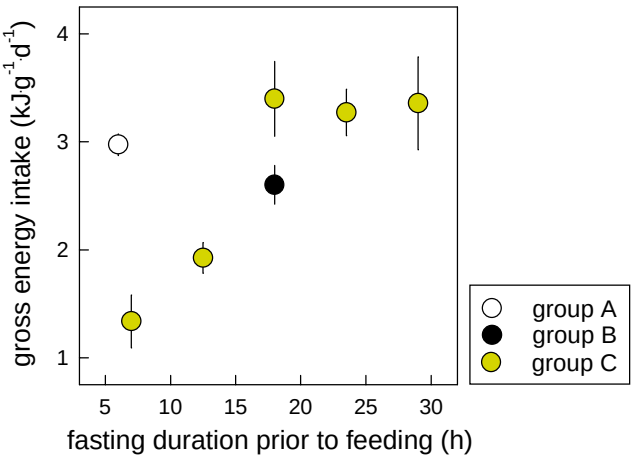


Figure 4. Relationship between daily gross energy intake, after correction for body mass ($kJ \cdot g^{-1} \cdot d^{-1}$), and number of hours fasting prior to feeding (h) in three groups of Japanese quail (A, B, and C). Values are means, and vertical lines indicate SDs. For more details, see figure 1.

level as in group A after 5 d. The growth efficiency in group C showed an irregular pattern: days with a high efficiency alternated with days of low or negative efficiency. Overall growth efficiency (total food intake over the whole experimental period per cage/total body mass increase over the same period per cage; %) varied significantly with treatment ($F_{2,22} = 22$, $p < 0.0001$). Overall growth efficiency was highest in group A (49 ± 2%), and did not differ significantly between group B (44 ± 2%) and group C (42 ± 3%).

In figure 6 we plotted the distribution of food intake, calculated as body mass increase per hour, for the first 6 h of food availability, regardless of fasting duration and time of food access in group C. Distribution of food intake varied significantly with treatment ($F_{2,37} = 129$, $p < 0.001$). Group B and C consumed significantly more food during the first 3 h of feeding than group A. During the last 3 h of food availability, group B had a higher intake than both other groups. No increase in food intake was visible at the end of the feeding period in group B and C.

DISCUSSION

This experiment showed that growing quail exposed to unpredictable feeding conditions gain less body mass than birds that receive the same amount of food per day but at fixed times. Quail chicks appear to respond mainly to these unpredictable conditions by adjusting their food intake in response to their energy needs as experienced on the moment itself, which is mainly determined by the length of the fasting period prior to feeding. Time of food availability also seemed to influence

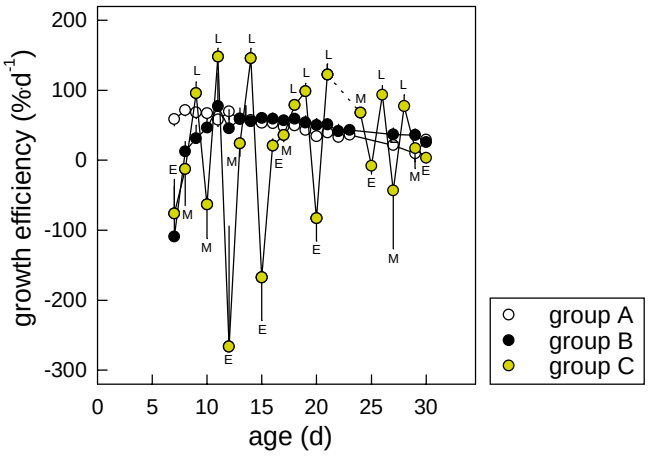


Figure 5. Mean growth efficiency (%·d^{-1}) as a function of age (d) in three groups of Japanese quail (A, B, and C). E, M, and L indicate feeding times in group C. Vertical lines indicate SDs. For more details, see figure 1.

feeding motivation in chicks: food arriving later in the day induced higher intake levels.

Growing quail subjected to unpredictable feeding circumstances had a lower body mass than birds offered the same amount of food per day but at fixed times (Fig. 2A). If a chick could anticipate fasting periods of unpredictable length or respond to the feeding conditions as experienced in the previous days, food intake should be consistently high and only depend on the size of the animal. In growing quail subjected to an unpredictable feeding regime food intake was highly variable: days of high intake alternated with days of low intake (Fig. 3A). It appeared that this fluctuation in food intake was determined by the variation in fasting duration prior to feeding (Fig. 4), indicating that the birds respond to their energy needs as experienced on the moment itself. We also showed that the birds of group C had a higher gross energy intake (GEI) when they had fasted for 18 h than birds that fasted for 18 h every day (Fig. 4). This could signify that the birds of group C do adjust to some extent to experience and consume more food than when they are subjected to the same fasting duration every day. Alternatively, these chicks, after an 18-h fast, have larger energy deficits, because of their irregular feeding pattern during the previous days. Another determinant of feeding motivation was the time at which food became available: chicks of group C, after an 18-h fast, had higher feeding activity levels at time L (Fig. 1), and also GEI seemed to increase when food arrived later in the day. Time of day is a well-established regulator of food intake and weight gain in a number of species. In free living birds eating is sometimes shifted to the end of the day. This may minimise the risk of predation (heavy birds are more vulnerable through decreased agility) or reduce the cost of flight (36,90,106,107). This applies to flying birds. The young quail investigated here are flightless, and furthermore highly domesticated, making a

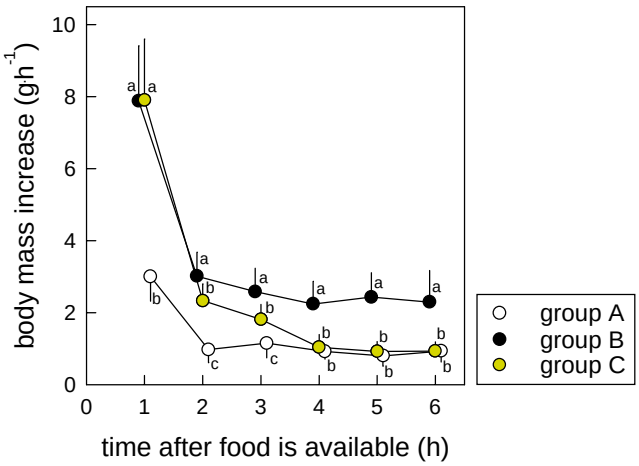


Figure 6. Distribution of food intake, calculated as body mass increase per hour ($g \cdot h^{-1}$), during the first 6 h of the feeding period in three groups of Japanese quail (A, B, and C). Values are means, and vertical lines indicate SDs. Means with the same letter within 'time after food is available' did differ not significantly (Tukey, $p > 0.05$) from each other. For more details, see figure 1.

relation of feeding behaviour with predation risk doubtful. The higher level of feeding activity at time L (Fig. 1) is therefore not likely due to a decreased 'perceived' threat of death from predation. More likely, spending part of the light period in anticipation of food arrival augmented feeding motivation. Although our design does not permit to distinguish between the effect of fasting duration and time of food arrival on feeding motivation, the results suggest that both factors contribute to overall feeding motivation.

In poultry restrictive feeding results in higher levels of locomotor and/or drinking activity compared to *ad lib.* conditions (64,161). In our study we did not find an effect of treatment on either total locomotor, feeding or drinking activity. All three types of activity showed merely a tendency to higher levels in group B and C. Locomotor activity in group C had a more irregular pattern when food arrival was delayed to later hours in the day compared to early in the day, and compared to the activity pattern of both groups with fixed feeding times (Fig. 1). This indicates that the chicks become restless during food unavailability, possibly searching for food. The daily distribution of movements above the feeder and the water container was affected by treatment. In group A more than 99% of all activity above the feeder occurred during the time of food availability, while in group B and C this percentage was 60–70%. Group C showed a large increase in feeding (and locomotor) activity at the start of food access that increased dramatically when food arrival was delayed to later hours in the day. Also group B showed an increase in both activity levels at the start of food availability, but less pronounced than in group C at time M and L (Fig. 1). These activity bouts far exceeded the initial activity bouts in group A (Fig. 1), and support the large bout in food intake during the first feeding hours in group B and C (Fig. 6). These birds

apparently had learned to eat as much as they could as soon as food became available. Birds in group C also showed an increase in activity above the feeder at the end of the feeding period when food arrived late in the day (time L). Broilers have been shown to be able to anticipate periods of food unavailability when this coincided with darkness: they consume more food at the end than at the onset of the feeding period (103). These birds were unable to anticipate a fasting period when exposed to continuous light: more food was consumed at the onset of the feeding period. Similarly, rats have a feeding peak at the end of darkness (= feeding period), which is advanced by advancing the light by 2 h, but not by advancing the onset of fasting by 2 h (75). In group B and C the end of food availability only coincided with darkness when food arrived late in group C, which may explain why under the other conditions chicks did not show an increase in food intake at the end of the feeding period. In group B and C a large part of the drinking activity (in group C even more than 50%) occurred outside the feeding period, while under *ad lib.* conditions drinking coincided mainly with feeding. This may be another indicator that the chicks are restless, possibly exploring their water containers in search of food. Because the total activity was not different between group B and C, a difference in energy expenditure is not likely to be an explanation for the difference in weight gain between these two groups.

GEI in both group B and C was reduced compared to group A, although the reduction was not proportional to the decrease in feeding time. The chicks in these groups learned quickly that food was present during a part of the day only. Already on the second day of the experiment the birds started to exploit their crop for temporary food storage that was mobilised when no food was available. In this way chicks could consume more food than expected on the basis of time reduction only. This strategy allowed group B to gain weight continuously after an initial drop during the first 2 d of the experiment (Fig. 2A). They increased both weight gain rate (Fig. 2B) and growth efficiency (Fig. 5) to the level of group A. Group C followed the same strategy, but with a different result for weight gain and GEI. Fasting duration in this group was not constant: days of only 7 h fasting alternated with days when the duration of fasting could be as long as 29 h. After fasting for only 7 h, the crop (and digestive tract) may still be largely filled with food. This would explain the low feeding motivation of these birds when feeding time E was preceded by feeding time L (Fig. 3). Consequently food intake was too low to cover the energy needs of the birds during the following fasting period and they lost weight. When the birds had been fasting for 18 h or more the crop (and digestive tract) may have been empty and feeding motivation was high (Fig. 4). In quail the control of feeding is concerned with emptying and filling of the digestive tract including the crop rather than with changing levels of circulating nutrients (160). The irregular distribution of food intake in group C resulted in large fluctuations of weight gain (days of low or no weight increase alternated with days of high weight increase) and a lower overall GEI as compared to group B. It is likely that both this difference in food consumed and the irregular distribution of intake resulted in a difference in overall weight gain between group B and C. In hamsters unpredictable food deprivation has a more suppressive effect on weight gain than shortage of food as such (69). Time of daily food arrival most likely

modulated the effect of crop (and digestive tract) contents on feeding motivation (Fig. 1).

Periodic daily food availability activates several processes in the body in anticipation of food arrival (34,83,167). In rats the gastrointestinal tract anticipates the time of feeding by increasing duodenal activity and levels of digestive enzymes 2–4 h prior to food availability (34). In this way a high efficiency of food digestion is accomplished. When food is offered at unpredictable times, the animal may not be able to synchronise feeding with anticipatory digestive processes, and digestion may be less efficient than in animals subjected to predictable feeding times. This may also partly explain the difference in weight gain between group B and C. In both humans and animals uncertain situations are known to influence the reproductive system (26,69,104). It is plausible that also our birds experienced the unpredictable feeding conditions as more trying, with a more suppressive effect on body mass. Uncertain situations, also when unrelated to food restriction, can have a detrimental effect on body mass (69,105,110).

In conclusion, the study showed that growing quail, subjected to either predictable or unpredictable restricted feeding conditions, quickly learn that feeding is only possible during a limited number of hours per day. To cope with these circumstances, the birds exploit their crop (and digestive tract) as a temporary storage place for food that is mobilised during the time that feeding is not possible. Restricted birds with fixed feeding times (group B) were thereby able to resume the same weight gain curve as group A with a delay of about 5 d (Fig. 2A). Restricted birds with unpredictable feeding times (group C) seemed to adjust their feeding strategy to the fasting duration prior to feeding. The young birds respond to their energy needs of the moment and seem neither to anticipate fasting periods, nor to respond to feeding conditions as experienced on previous days. They thereby showed a further retardation in body mass compared to quail chicks on a daily constant restricted feeding regime. This response to energy needs was modulated by time of food arrival: motivation was higher when feeding time was delayed to later hours in the day. Predictable daily variations in food supply offer the opportunity for better adaptive adjustments of food intake and weight gain than unpredictable variations.

CHAPTER 6

EFFECT OF DAY LENGTH ON THE RESPONSE OF PROTEIN SYNTHESIS TO FASTING AND FEEDING IN GROWING JAPANESE QUAIL (*COTURNIX C. JAPONICA*) CHICKS

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Submitted

ABSTRACT

In this study we investigated the effect of day length on mixed protein fractional synthesis rates (K_S) in fast growing 14- and 21-d old Japanese quail (*Coturnix c. japonica*) subjected to either a long, 18L:6D (LD), or short day length, 6L:18D (SD), with *ad lib.* food during the light period. We measured mixed protein fractional synthesis rate of pectoralis muscle, liver, and heart after an overnight fast and after 2 h food access at dawn. Protein synthesis was also measured in LD-quail after an 18-h fast, and after 2 h of food access at dawn after an 18-h fast, to correct for the difference in fasting duration between day lengths. Protein synthesis was measured by a flooding dose of L-[1-^{13}C] labelled leucine. We tested the following hypotheses: chicks habituated to short day lengths are able (1) to maintain high fasting protein synthesis rates, and (2) to deposit more dietary amino acids into body protein during feeding than birds subjected to long day lengths. Results revealed that exposure to a short day length (and consequently long nocturnal fasts) induced a pronounced rise in tissue protein synthesis rates with food availability. This increase was absent in birds subjected to a long day length. Being habituated to a short day length did not induce high protein synthesis rates at night and/or increase the measured rates of protein synthesis during feeding. Protein synthesis rates were highly flexible, and responded rapidly to *ad hoc* changes in food supply. We conclude that day length, by determining the daily period in which feeding can occur, has a major effect on protein synthesis rates. This effect will determine the overall growth chicks are able to achieve when exposed to different day lengths.

INTRODUCTION

Growth in juvenile animals is affected by day length (17,19,162). This effect is produced by the influence of day length on daily energy intake and daily energy expenditure (17,19,28,162). Growth in young animals is mainly increase in body protein, achieved by maintaining protein synthesis at a higher rate than protein breakdown (188). It seems therefore feasible that day length, via time available for energy intake and energy expenditure, affects protein turnover by influencing synthesis and/or breakdown rates, and thus net growth.

Daily patterns in food intake induce daily variations in protein turnover in diurnal animals: daytime feeding results in protein gain, and night-time fasting in protein loss (32,112,113,119,141). For net growth of tissues to occur, as in growing and lactating animals, the daytime deposition of protein should exceed nocturnal protein losses (50). Exposure to short day lengths results in long nocturnal periods of fasting and large amounts of food consumed during short light periods. During these short feeding periods a large amount of amino acids becomes available to the body. These dietary amino acids should be rapidly used for protein synthesis to cover immediate protein needs (repair, growth and maintenance), and/or as a protein store for use in subsequent fasting periods. When the supply of amino acids exceeds the protein synthesis capacity of the body, this excess will be oxidised (54,112,113,188). During the nocturnal fast protein synthesis rates will drop, and with prolonged fasting rates may even fall below breakdown levels. Fasting for a period of 2–4 d reduces protein synthesis rates considerably in different tissues and whole body (29,108,109,117). Even shorter-term fasting (8–18 h) has been shown to reduce protein synthesis rates (42,49,141,199). Thus, long nocturnal fasting periods may be a limiting factor for overall growth.

In most of the studies mentioned above, fasting and feeding were imposed to investigate the acute effects on protein synthesis rates. There is not much information concerning the mechanisms whereby protein synthesis rates are affected by fasting and feeding periods that return daily over a longer time span. Are animals habituated to short daily feeding periods, and consequently long nocturnal fasts, able to maintain protein synthesis rates during the overnight fast at a rate higher than those in animals that are not conditioned to prolonged fasts? In addition, are they able to deposit a larger amount of their dietary amino acids consumed during a short feeding period into body protein compared to animals that can obtain food during the main part of the 24-h period? The capacity for deposition of protein during feeding will determine the extent of amino acid losses that are lost for growth. Our study addresses these questions. We investigated the effect of day length on the response of protein synthesis rates to fasting and feeding in a fast growing strain of Japanese quail (*Coturnix c. japonica*) subjected to either a long (18L:6D) or short day length (6L:18D) with *ad lib.* food during the light period. We measured the fractional synthesis rate (K_S) of tissue mixed protein after an overnight fast and after 2 h food access at dawn. K_S was measured of two different muscle tissues (skeletal and cardiac muscle) and liver. Skeletal muscle and liver were chosen because both tissues contribute considerably to whole-body protein synthesis. In all organisms where it has been

measured, hepatic K_S is high, while muscle, with a much lower K_S, contributes by its large size of the protein pool (108,188). Cardiac muscle was included because of its continuous, rhythmic activity, which makes it likely less sensitive to fasting and food intake. Japanese quail were used, a species with the fastest growth rate of the family Phasianidae (151). We have shown that this species is highly responsive to variations in day length and food availability (16-18). In the present study all measurements were conducted on birds of 2–3 weeks of age, the period of highest absolute body weight gain (17).

METHODS

Animals and housing

Japanese quail (*Coturnix c. Japonica*) neonates, of a strain selected for maximum body mass at the age of 5 weeks, were obtained from a commercial quail farm (N.V. Nouwen, Lommel, Belgium). Until 6 d of age birds were kept in wooden cages ($l \times b \times h$: 67×39×44 cm^3) with sawdust bedding at conditions of continuous light and *ad lib.* access to quail starter food and water, to ensure maximum possible body mass gain. A 40 W heating lamp was placed in each cage to provide a temperature gradient sufficient for selection of the preferred temperature by the chicks. At 6 d of age the chicks were assigned to either a long, 18L:6D (LD), or short day length, 6L:18D (SD; lights on at 0900 hours (MET)), with *ad lib.* food during the light period. The birds were housed in the same cages as mentioned above but with a wire bottom. The 40 W heating lamp was gradually removed to allow the ambient temperature to decrease to room temperature (~21°C) within 3 weeks of age. At 6 d of age, the chicks were permitted to habituate to the experimental conditions and permitted to eat *ad lib.* during the whole 24-h period. The experiment started at 7 d of age. Throughout the experimental period a pellet-diet (Institute for Animal Science and Health, ID-DLO, The Netherlands) containing 27.7% (w/w) crude protein and 17 $kJ \cdot wet\ g^{-1}$ (gross energy content as determined by bomb calorimetry; own measurement) was fed. Water was always freely available.

Experiment 1: time course of enrichment in plasma, liver and muscle

To develop a valid protocol for measuring tissue mixed protein fractional synthesis rates (K_S; % of protein mass synthesised per h) with the use of the flooding dose technique in fast growing quail, we examined the time course of [^{13}C-]Leu enrichment in the free pools of plasma, pectoralis muscle and liver, and protein bound pools of pectoralis muscle and liver after administrating a flood of L[1-^{13}C]Leu (51). Growing quail of 14 d of age (85 ± 15 g), subjected to a long day length, received a single intramuscular (i.m.) injection (1 $ml \cdot 100^{-1}$ g body mass containing 20% w/w L-[1-^{13}C]leucine) in the left pectoralis muscle at dawn after an 18-h fast. We decided to use i.m. injection to make the protocol comparable to other work on birds (120). After 5 ($n = 7$), 15 ($n = 4$), 30 ($n = 4$), and 60 ($n = 4$) min, taken as the time elapsed from the moment that the whole solution was injected, birds were sacrificed by immersion in liquid nitrogen. Whole liver and a sample of the right pectoralis muscle were

Table 1. Characteristics (g) of 14- and 21-d old Japanese quail exposed to either a long (LD; 18L:6D) or short day length (SD; 6L:18D), that were either fasted for 6 or 18 h, or had 2 h food access at dawn. Values are means ± SDs.

	14 d				21 d	
mass (g)	fasted for 6 h	food after 6-h fast	fasted for 18 h	food after 18-h fast	fasted for 18 h	food after 18h-fast
LD						
n	8	8	8	8	–	–
body mass	95.5 ± 10.5	99.1 ± 11.9	94.9 ± 10.9[†]	96.1 ± 14.8	–	–
muscle	6.57 ± 10.9	6.58 ± 1.09	7.84 ± 1.15	7.29 ± 1.17	–	–
liver	2.83 ± 0.50	2.97 ± 0.47	2.61 ± 0.48	2.78 ± 0.51	–	–
heart	0.83 ± 0.14	0.81 ± 0.14	–	0.71 ± 0.15	–	–
SD						
n	–	–	7	6	6	6
body mass	–	–	51.0 ± 7.0	65.1 ± 11.0	110 ± 15.8	126 ± 17.0
muscle	–	–	3.04 ± 0.86	3.90 ± 0.65	8.65 ± 0.64	8.57 ± 1.82
liver	–	–	1.33 ± 0.24	2.22 ± 0.51	2.41 ± 0.43	3.54 ± 0.33
heart	–	–	0.48 ± 0.05	0.60 ± 0.14	0.87 ± 0.10	0.91 ± 0.27

[†] Data from experiment 1.

immediately removed and washed in ice cold saline (9 g sodium chloride/L) to minimise blood contamination. The tissues were then rapidly frozen in liquid nitrogen, weighed (to 0.0001 g), and stored at -70°C until analysis. The whole procedure of tissue harvesting took less than 2 min. Just before sacrificing the chicks, we collected blood from the right wing vein in heparinized tubes, which were then centrifuged (2600 rpm, 15 min) for plasma collection. Plasma was stored at -70°C till analysis. A group of three birds was used for baseline measurements of L-[1-^{13}C]leucine enrichment in each tissue taken.

Experiment 2: K_S in the fed and fasted state related to day length

We measured K_S of pectoralis muscle, liver and heart of 14-d old SD- and LD-quail after an overnight fast and after 2 h food access at dawn. Because protein turnover rate is body mass related (55,111,188), we repeated the experiment in 21-d old SD-chicks. Body mass of SD-chicks at that age is more comparable to that of 14-d old LD-chicks (Table 1). We also measured K_S in 14-d old LD-chicks fasted for 18 h after 2 h food access, comparable to the daily fasting duration of SD-chicks. To measure K_S under all conditions, we injected chicks as described in experiment 1, and sacrificed them at either 15 or 30 min after injection. Whole liver, a sample of the right pectoralis muscle, and whole heart were taken and processed as described above. Three animals were used for baseline measurements of L-[1-^{13}C]leucine enrichment in each tissue taken.

Table 2. (A) The ratio of muscle and liver free pool enrichment relative to plasma enrichment over time and (B) mixed protein fractional synthesis rates (K_S; %·h^{-1}) of muscle and liver calculated over different time periods in 18-h fasted 14-d old Japanese quail after a flooding dose of L-[1-^{13}C]leucine. Values are means ± SDs.

A.	ratio		**B.**	K_S (%·h^{-1})	
time (min)	muscle	liver	period (min)	muscle	liver
5	0.79 ± 0.11	1.06 ± 0.14	5-15	0.47 ± 0.18	2.21 ± 0.68
10	0.79 ± 0.08	0.88 ± 0.07	5-30	0.49 ± 0.12	1.46 ± 0.36
15	1.06 ± 0.04	1.05 ± 0.07	5-60	0.68 ± 0.45	1.70 ± 0.74
30	1.05 ± 0.15	0.87 ± 0.03	15-30	0.59 ± 0.13	3.61 ± 1.20
60	0.61 ± 0.28	0.94 ± 0.45	15-60	1.04 ± 0.68	3.31 ± 1.44
			30-60	2.34 ± 1.55	8.63 ± 3.76

Tissue analysis

A 100 to 200 mg sample of tissue was first homogenised in liquid nitrogen using a mortar and pestle. The tissue was mixed with 3 ml ice-cold 0.2 M perchloric acid and after centrifugation (2800 × *g*), the supernatant, containing the tissue free amino acids, was decanted and neutralised with potassium hydroxide. The amino acids from this solution were purified by ion-exchange chromotogaphy, dried, and treated with 50 µl of pyridine and 50µl of methylsilyl*tert*-butylsilyltetrafluoroaretamide (MTBSTFA). Labelling of the tissue free leucine enrichment, as tert-butyl dimethylsilyl derivative, was then measured by gas chromatography-mass spectrometry, MD 800 (Fisons Instruments, UK) operated in selected ion monitoring mode.

To determine the bound tissue protein enrichment, the pellet obtained from the treatment of the tissue with perchloric acid was processed to remove RNA and DNA, and then subjected to hydrolysis in 3 ml 6 M hydrochloric acid at 110°C for 15–18 h. Released amino acids were purified by ion-exchange chromatography, dried, and the leucine was collected by preparative gas chromatography (172). Enrichment of the tissue protein leucine was determined by isotope ratio mass spectrometry (Europa Scientific Instruments 2020) after liberation of the carboxyl carbon as $^{13}CO_2$ by the ninhydrin reaction (172).

Calculations and data analysis

Mixed protein fractional synthesis rate (K_S; %·h^{-1}) was calculated as

$$K_S = [(E_B / E_A) \times t^{-1}] \times 100$$

where E_B is the enrichment of the tissue protein-bound leucine, E_A is the enrichment of the tissue-free leucine, and t is the time of labelling in hours (51,109). We used the mean enrichments of the free and bound pool over the different time periods (x_1–x_2 min; Table 2) after administrating the flood. To this end we calculated, for each individual quail at x_2 min, its own value for E_A and E_B. For this, we multiplied each free and bound amino acid enrichment at x_2 min ($E_{A(x2)}$ and $E_{B(x2)}$, respectively) by the value $\hat{E}_A/\hat{E}_{A(x2)}$ and $\hat{E}_B/\hat{E}_{B(x2)}$, respectively. $\hat{E}_A$ and $\hat{E}_B$ are the mean free and bound amino

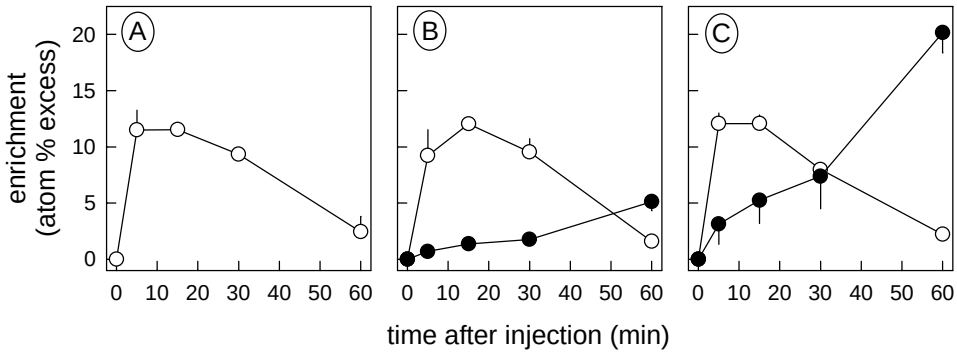

Figure 1. Time-related changes in enrichment (atom % excess; mean ± SD) of L-[1-^{13}C]leucine in the free (open symbols) and bound pool (* 100; closed symbols) of (A) plasma, (B) pectoralis muscle, and (C) liver in 18-h fasted 14-d old Japanese quail after a flooding dose of L-[1-^{13}C]leucine.

acid enrichments over x_1–x_2 min, and $\hat{E}_{A(x2)}$ and $\hat{E}_{B(x2)}$ the mean values at x_2 min, obtained from the group values (51).

Data are expressed as means and inter-individual standard deviations. Student's *t* test was used to test for differences between group means and to test for the effect of feeding state (fasted versus fed) on body mass and tissue K_S within day length (SPSS Inc., 1988). We also applied the Student's *t* test to investigate the effect of fasting duration within the LD-group on tissue K_S (SPSS Inc., 1988). Differences in mean K_S between tissues within day length were subjected to posthoc analysis by Tukey's "honestly significant difference" test, after an effect of day length on tissue K_S was ascertained by ONEWAY analysis. Analysis of variance (ANOVA) was used to test for the effect of day length, feeding state, and day length × feeding state interaction on tissue K_S. We applied a two-tailed significance level of $p<0.05$ in all tests.

RESULTS

Experiment 1

The enrichment of the free pools (E_A; atom % excess) increased rapidly after injection (Fig. 1). In plasma and liver the highest level was reached after 5 min, with no significant decrease over the next 10 min. In muscle the highest level was achieved after 15 min. Over the last 45 min, E_A decreased in all tissues. In the protein bound pool of muscle and liver we observed a linear increase in enrichment (E_B) over the first 30 min. E_B increased steeply in both muscle and liver 30–60 min after injection. In table 2A we listed the ratio for muscle and liver free pool enrichment relative to the enrichment of plasma over the time course of the experiment. It is evident from these results that flooding conditions were reached after 5 min for liver. Flooding conditions for muscle were only reached after 15 min, but were maintained for the period chosen for protein measurements (see below; Table 2A). Calculated mixed

protein K_S for the different time intervals are presented in table 2B. Because of the time course of E_A and E_B in the different tissues (Fig. 1) and the tissue/plasma enrichment ratio (Table 2A), we decided to take the 15–30 min-interval for the experimental protocol.

Experiment 2

Body mass

Table 1 shows different characteristics of 14- and 21-d old SD- and LD-quail that were either fasted for 6 or 18 h, or had access to food for 2 h at dawn. Body mass varied only significantly with feeding state in 14-d old SD chicks ($T_{21} = 11$, $p < 0.05$; Table 1). Because of this feeding effect we tested for a day length effect on body mass by comparing fasted 14-d old SD-chicks with equal aged LD-chicks that were either fasted or fed. Body mass varied significantly with day length: SD-chicks weighed 47% less than LD-chicks (51.0 ± 7.0 g versus 96.4 ± 11.7 g, respectively; $T_{39} = 9.9$, $p < 0.001$). SD-chicks of 21 d, irrespective of feeding state, weighed significantly more (18%) than 14-d old LD chicks: 118 ± 17.6 g and 96.4 ± 11.7 g, respectively ($T_{42} = -4.8$, $p < 0.001$).

Mixed protein fractional synthesis rates (K_S)

In table 3 we listed the mean K_S of pectoralis muscle, liver, and heart for the different experimental conditions. Compared to the 6-h fasting state the measured K_S of pectoralis muscle, liver, and heart were not affected by 2 h food access in LD-chicks (Table 3). In SD-chicks, irrespective of age, pectoralis muscle K_S was 3 times higher after 2 h food access at dawn than in the fasted state: 1.92 ± 1.02 %·h^{-1} versus 0.64 ± 0.23 %·h^{-1}, respectively ($T_{5.49} = -3.0$, $p < 0.05$). Liver K_S was increased 1.6 times: 8.61 ± 2.04 %·h^{-1} and 5.52 ± 1.03 %·h^{-1}, respectively ($T_{10} = -3.3$, $p < 0.01$). Heart K_S values did not significantly vary with feeding state in SD-chicks. Because none of the tissue K_S values varied with nutritional status in the LD-group, we compared mean K_S between the different tissues irrespective of feeding state. In the LD-group K_S was highest in liver (6.25 ± 1.16 %·h^{-1}) and did not differ statistically between pectoralis muscle (1.61 ± 0.36 %·h^{-1}) and heart (2.34 ± 0.42 %·h^{-1}; $F_{2,21} = 91$, $p < 0.0001$). In SD-quail, irrespective of age and after an 18-h fast, K_S varied also significantly between the different tissues ($F_{2,15} = 97$, $p < 0.0001$): K_S was highest in liver (5.52 ± 1.03 %·h^{-1}) and lowest in pectoralis muscle (0.64 ± 0.23 %·h^{-1}). Heart K_S was intermediate between these two values (1.72 ± 0.34 %·h^{-1}). In the fed state, the difference in K_S between pectoralis muscle and heart disappeared (1.92 ± 1.02 %·h^{-1} and 2.04 ± 0.68 %·h^{-1}, respectively), while liver K_S remained highest (8.61 ± 2.04; $F_{2,15} = 47$, $p < 0.0001$).

To test for an effect of day length on tissue K_S, we compared for each tissue separately 14-d old LD-chicks with 21-d old SD-chicks, because of more comparable body mass (Table 1). For this we entered day length, feeding state, and day length × feeding state interaction in one ANOVA. Pectoralis muscle and heart K_S varied only significantly with day length (both $F_{1,13} = 11$, $p < 0.01$): K_S in both tissues was higher in

Table 3. Mixed protein fractional synthesis rates (K_S; %·h^{-1}) of different tissues of 14- and 21-d old Japanese quail exposed to either a long (LD; 18L:6D) or short day length (SD; 6L:18D), that were either fasted for 6 or 18 h, or had 2 h food access at dawn. K_S was measured by a flooding dose of L-[1-^{13}C]leucine. Values are means ± SDs.

	14 d				21 d	
tissue	fasted for 6 h	food after 6-h fast	fasted for 18 h	food after 18h-fast	fasted for 18 h	food after 18h-fast
LD						
n	4	4	4	4		
muscle	1.57 ± 0.50	1.64 ± 0.24	0.59 ± 0.13[†]	1.39 ± 0.40	–	–
liver	6.04 ± 1.37	6.46 ± 1.07	3.61 ± 1.19	9.20 ± 3.71	–	–
heart	2.33 ± 0.57	2.35 ± 0.30	–	2.12 ± 0.84	–	–
SD						
n			3	3	3	3
muscle	–	–	0.66 ± 0.22	2.59 ± 1.06	0.62 ± 0.28	1.24 ± 0.31
liver	–	–	6.20 ± 1.00	10.0 ± 1.93	4.85 ± 0.50	7.18 ± 0.72
heart	–	–	1.92 ± 0.37	2.24 ± 1.00	1.52 ± 0.14	1.85 ± 0.23

[†] Data from experiment 1.

the LD-group for both fed and fasted state. Liver K_S was not significantly affected by feeding state or day length.

Subjecting LD-chicks to an 18-h fast reduced K_S of pectoralis muscle and liver significantly more than a 6-h fast (T_6=-3.8, $p<0.01$ and T_6=-2.7, $p<0.05$, respectively; Table 3). After an 18-h fast in LD-chicks, in contrast to a 6-h fast, 2 h food access significantly stimulated K_S in both pectoralis muscle (T_6=-3.8, $p<0.01$) and liver (T_6=-2.9, $p<0.05$). To test for an effect of day length on pectoralis muscle and liver K_S corrected for hours fasting, we compared 14-d old LD-chicks fasted for 18 h with fasted 21-d old SD-quail, because of more comparable body mass (Table 1). To this end, day length, feeding state, and day length × feeding state interaction were entered in one ANOVA. Pectoralis muscle ($F_{1,13}$=22, $p<0.005$) and liver K_S ($F_{1,13}$=12, $p<0.01$) varied only significantly with feeding state: K_S of both tissues was higher in the fed compared to the fasted state.

DISCUSSION

This experiment showed that exposure to a short day length (and consequently long nocturnal fasts) resulted in a pronounced rise in tissue mixed protein synthesis rates during food availability. This increase was absent in birds conditioned to a long day length and hence longer periods of food availability. Being habituated to a short day length did not appear to induce any compensatory high tissue protein synthesis rates at night and/or increase the deposition of dietary amino acids during feeding.

Day length had a major effect on body mass in fast growing quail (Table 1). Animals subjected to a short day length had a smaller average body mass at 2 weeks of age compared to birds subjected to a long day length. This was due to the

reduction in time available for food intake and consequently protein intake. We showed earlier that growing quail subjected to daily feeding periods of 6 h consumed on average 29% less energy than LD-birds of equal body mass (17). Day length reduction would be expected to have a profound influence on body protein turnover (and consequently growth). To measure the likely effects of day length on protein synthesis we first demonstrated that it is possible to use the flooding dose method to measure tissue protein synthesis in fast growing birds. The flooding dose method is a widely adopted technique to measure whole-body and tissue protein synthesis rates in small animals (50). It is a convenient method for measuring acute changes in tissue protein synthesis as can occur, for example, after feeding (42,49,50). Flooding dose experiments in rats (51,109,129), dogs (71), and birds (120) show a linear decline in free pool enrichment (E_A) with time shortly after injection of the label. This was not apparent in our study, most likely due to a difference in the route of label administration. In rat studies the label is usually injected intravenously, leading to a rapid rise of plasma and tissue free pool enrichment (to a maximum level within 2 min), that drops curvi-linearly afterwards. In a study with the White-crowned sparrow (*Zonotrichia leucophrys gambelii*) a large dose of ^{3}H-phenylalanine was injected in the pectoralis muscle, similar to our study (120). In these birds free pool enrichment of muscle and liver reached their highest levels within 5 min after injection. The pectoralis muscle in quail contains mainly white fast-twitch glycotic fibres that are not highly vascularized (66). Because of this poor vascularization the movement of the injected label from the left pectoralis muscle into the bloodstream and then into the tissue free amino acid pools may be attenuated. This may explain the slow rise with time in free pool enrichment of pectoralis muscle in our study compared to experimental protocols using intravenous or better vascularized routes. In plasma and liver we observed the highest E_A levels within 5 min. After 15 min E_A decreased linearly in all compartments (Fig. 1). The bound pool enrichment (E_B) increased linearly with time up to 30 min after injection (Fig. 1). Thereafter, E_B increased steeply, while at the same time the enrichment of the free pools had started to reach low values (± 13% of maximum level). This rise in incorporation rate may be the result of a stimulation of protein synthesis due to the flood (149,170,171), and/or slower transit times for the tracer into protein relative to rates of protein synthesis and the time course of the flooding dose. Because of the slow rise in muscle E_A and the steep increase in E_B 30 min after injection of the label, we calculated protein fractional synthesis rates (K_S) using measured and estimated enrichments 15 and 30 min after injection. Also, because we demonstrated clearly that at these time points flooding conditions were reached in both tissues (Table 2A). Mixed protein K_S calculated over this time period were comparable to those reported for other bird species (97,98,120).

Using this method we showed that exposure to short day lengths (and consequently long overnight fasts) resulted in (a) a pronounced decrease in tissue mixed protein synthesis rates during the overnight fast and (b) a steep rise in measured synthesis rates during feeding (Table 3). It has been suggested that moulting birds, that face high energy demands, may undergo pronounced daily cycling of protein, involving net protein degradation during the night when exposed to long

overnight fasts (≥ 8 h) and net synthesis by day (119). The young birds investigated here were fast growing animals, indicating that energy demands are also liable to be high. SD-chicks are therefore likely to show a discontinuous 24-h growth curve: net protein gain during the day alternates with net protein loss during the night. Overall, birds were clearly in a net positive protein balance as they grew over the experimental period. Quail chicks subjected to overnight fasts of 6 h had fasted tissue K_S values comparable to fed rates (Table 3): birds are able to maintain a constant level of protein synthesis throughout the 24-h period. A daily fasting duration of 6 h also ensured chicks of relatively high tissue K_S values throughout the 24-h period (Table 3). These findings suggest that LD-chicks are able to grow continuously throughout the 24-h period at a high rate. We are aware that we only studied protein synthesis rates. Because growth in protein is the difference between protein synthesis and protein breakdown (188), an increase in K_S only results in increased tissue protein growth when breakdown levels are increased less, or even reduced during feeding. However, it is possible to predict that protein breakdown rates have likely fallen either during or immediately after feeding. In mice it has been shown that re-feeding for 1 h after an 18-h fast resulted in a decrease in muscle protein breakdown and an increase in muscle protein synthesis (199). The inhibition of protein degradation rates due to feeding has also been shown in human studies (54,133). It is therefore likely that in our experiment too the increase in K_S, and any decrease in protein breakdown, due to feeding has resulted in an increase in tissue protein growth. Increase in protein synthesis rates due to feeding depends on the amount of food consumed (188). We have shown earlier that feeding during the first 2 h of food availability is largely increased in SD-chicks, and only minimally in LD-chicks (18). This may explain the steep rise in muscle and liver K_S at dawn in SD-chicks. The fact that no stimulation of protein synthesis occurred in LD-chicks after 2 h food access may be due to a low food intake level during this period.

LD-chicks subjected experimentally to an 18-h fast had comparable tissue protein synthesis rates at dawn as birds habituated to repeated 18-h fasts (Table 3). We had thus no support for our first hypothesis that birds habituated to 6-h feeding periods develop adaptive mechanisms by which they can maintain high protein synthesis rates during daily periods of prolonged fasting. We can however not exclude that the drop in K_S during the overnight fast was delayed in SD-chicks compared to 18-h fasted LD-chicks, resulting in less protein loss during the nocturnal fast. We have shown that growing quail habituated to a long daily overnight fast were able to offset (partially) the effects of the fast by exploiting their crop as a temporary place for food storage (17,18). In this way food is present in the gastrointestinal tract during a large part of the night, as was evident from a high RQ (17). LD-chicks subjected experimentally to an 18-h fast will not have developed this strategy of using their crop as a food store. The loss of protein during the night may therefore be less in SD-chicks than LD-chicks subjected to an 18-h fast. LD-chicks that were re-fed for 2 h after an 18-h fast showed a comparable stimulation of muscle and liver K_S as 21-d old SD-chicks during feeding (Table 3). We hypothesised that SD-chicks, conditioned to repeated long daily overnight fasts, would show a higher increase in tissue K_S during feeding than LD-chicks. Because feeding is restricted to only 6 h of the 24-h period, a

large amount of amino acids become available to the body in a relatively short period. For efficient growth to occur these dietary amino acids must be rapidly and efficiently deposited as body protein with minimal oxidative losses. Adopting this strategy, chicks could enlarge their amino acid store for periods during which no feeding can occur. Muscle K_s in particular may be responsive. Muscle is known to contain the largest reserve of mobilizable protein in the body (188), and has been suggested to perform a storage function in meeting amino acid demands in the postabsorptive state (120). However, we were unable to find evidence for this hypothesis (Table 3). Results indicate that protein synthesis rates are highly flexible and respond quickly to *ad hoc* changes in food supply. Although day length did not affect tissue K_s after an 18-h fast, the increase in liver K_s due to feeding tended to be higher in LD-chicks after an 18-h fast than in 21-d old SD-chicks: relative increase in liver K_s was 2.5 and 1.5, respectively (Table 3). As mentioned above LD-birds subjected experimentally to an 18-h fast are not likely to anticipate a long fasting period by food storage. Feeding motivation at dawn will therefore be even higher in these birds than in SD-chicks, resulting in a large increase in liver K_s after 2 h food access (Table 3). This was only visible in liver, because this organ receives dietary amino acids directly from the portal vein and is therefore highly sensitive to feeding (177).

Feeding had a significant effect on liver and pectoral muscle K_s after an 18-h fast as has been demonstrated in other studies after prolonged overnight fasts (29,42,49,141,148,198,199). Feeding however did not influence heart K_s. Heart is a rhythmically active organ that supplies tissues with nutrients and that removes waste products from the body. It is vital for life and growth, and can not afford to function less due to the absence of dietary nutrients. This relative insensitivity to fasting and feeding has very likely consequences for the metabolism and growth rate of fast growing birds.

Whole-body absolute protein turnover rates in mature animals of different species are positively related to metabolic mass (body mass to the power 0.75; 55,111,146,188,189). Protein turnover rates per unit mass on the other hand are negatively related to metabolic mass (body mass to the power -0.25). In our study there was a significant difference in body mass between 14-d old LD- and 21-d old SD-chicks (96 g versus 118 g). This may have interfered with the results. However, based on the relationship between protein turnover rate per unit mass and metabolic mass we calculated that the difference in body mass between the two groups could only account for $\sim 5\%$ of the change in mass corrected protein turnover rates. This is a marginal effect considering the scale of differences observed after feeding and the variation within the groups. It is therefore unlikely that this difference in body mass has affected the comparison between tissue K_s values. This relationship also explains the high K_s values in the 14-d old SD-group that had a significantly lower body mass than equal aged LD-chicks (Table 1, 3).

In conclusion, the results showed that day length affects protein synthesis rates by determining the daily duration of food access in diurnal animals. In this way day length will determine the level of overall growth juvenile animals are able to achieve when exposed to different day lengths.

CHAPTER 7

VALIDATION OF THE DOUBLY LABELLED WATER METHOD IN JAPANESE QUAIL (*COTURNIX C. JAPONICA*) CHICKS: IS THERE AN EFFECT OF GROWTH RATE?

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Submitted

ABSTRACT

The doubly labelled water (DLW) method was validated against respiration gas analysis in growing Japanese quail chicks (between 1 and 3 weeks of age) as well as in adults (7 weeks of age). We compared a strain selected for high growth rates (broilers, $n = 18$) with a normal growing strain (layers, $n = 18$). Relative rates of body mass increase of the chicks during the measurements ranged from -13.8 %·d^{-1} to 23.1 %·d^{-1}. When employing a single pool model (Eqn 34 of (88)), it was found that the relative error of the DLW method was sensitive to assumptions concerning fractional evaporative water loss. The best fit was obtained after taking a fractional evaporative water loss value of 0.33. When applying this value for all chicks, strain, relative rate of body mass gain during the measurement, and age did not significantly contribute to the explained variance. When employing a two pool model, the DLW method significantly underestimated the true rates of CO_2 production at all assumed levels of fractional evaporative water loss (0, 0.25, and 0.50).

INTRODUCTION

Since the publication of the seminal paper of (88), the doubly labelled water (DLW) method has frequently been used to measure the rate of carbon dioxide production in free-living adult birds and mammals (174). The application of this method is based on the assumption that, following administration of a pulse-dose of ^{2}H and ^{18}O, ^{2}H atoms leave the body water pool exclusively as water, and ^{18}O only as water and carbon dioxide gas (88). The DLW method has been validated in adult birds of about 15 species over a size range spanning three orders of magnitude (for the most recent review, see (174)).

In growing birds the application of the DLW method has been hampered by uncertainties concerning the routes of disappearance of both labels from the body water pool. For growing birds, it has been questioned whether ^{2}H and ^{18}O atoms do not only leave the body water pool as water and carbon dioxide gas, but also via incorporation in growing tissues. If incorporation does occur, and if the rates of incorporation differ between ^{2}H and ^{18}O, the error in the estimated rate of CO_2 production could be up to 25% (193). A first validation study of the DLW method in growing birds was published on the Arctic Tern (*Sterna parasidaea*), a semi-precocial species (79). It was found that the DLW method underestimated the true rate of CO_2 production by 10.3% on the average, which may suggest that differential incorporation of ^{2}H and ^{18}O does occur in this species during growth. These authors employed Eqn 35 of (88) to calculate the rate of CO_2 production from DLW measurements to correct for fractionation of the heavy isotopes. This equation assumes that a fraction of 0.50 of the water efflux is lost through evaporative pathways. It was demonstrated that the fit of the Arctic Tern validation study could substantially be improved by assuming a fraction of 0.20 instead of 0.50 (183). An identical result was obtained in growing precocial shorebird chicks (Black-tailed Godwit (*Limosa limosa)* and Northern Lapwing (*Vanellus vanellus*); (183)). After employing Eqn 35 of (88), it was found in these birds that the DLW method underestimated the true rate of CO_2 production by 11.6% on average, and that the best fit was obtained at a fractional evaporative water loss of 0.13. The authors did not find a correlation between the relative rate of body mass increase of the chick during the measurement and the error of the DLW method (183).

In spite of these observations, differential incorporation of ^{2}H and ^{18}O in growing tissues in young birds cannot be ruled out. The effect of this process can be obscured in a mathematical sense. For instance, by the policy of fitting values for the fractional evaporative water loss instead of relying on direct measurements. To further investigate the effect of growth rate on the application of the DLW method, we performed a validation study in growing precocial Japanese quail (*Coturnix c. japonica*) of two different strains. We employed one strain selected for high postnatal growth rate and a normal growing strain. If differential incorporation of ^{2}H and ^{18}O does play a role during the application of the DLW method, and if this effect is related to growth rate, we would expect that in chicks of the fast growing strain the DLW method has a stronger tendency to underestimate the 'true' rate of CO_2 production. To investigate the effect of the relative rate of body mass increase within each strain,

validation experiments were performed throughout juvenile development until sexual maturity. As far as we are aware, this is the first study in which the DLW method has been validated in a species over a wide range of developmental stages.

METHODS

Animals and housing

Japanese quail (*Coturnix c. japonica*) neonates of a fast (broilers) and normal growing strain (layers) were obtained from a commercial quail farm (N.V. Nouwen, Lommel, Belgium). Until the age of 6 d the birds were kept in wooden cages (*l*×*b*×*h*: 67×39×44 cm^3) with sawdust bedding in continuous light and *ad lib.* access to quail starter food and water, to ensure maximum possible body mass gain. A 40 W heating lamp was placed in each cage to provide a temperature gradient sufficient for selection of the preferred temperature by the chicks. This lamp was gradually raised and finally removed to allow the ambient temperature to decrease to room temperature (~21°C) within 3 weeks of age. At 6 d of age the light regime was switched to 18L:6D (lights on at 0900 hours MET). From this age onwards birds had *ad lib.* access to water and food pellets (Institute for Animal Science and Health, ID-DLO, The Netherlands) containing 27.7% (w/w) crude protein and 17 $kJ \cdot wet\ g^{-1}$ (gross energy content as determined by bomb calorimetry; own measurement).

Experimental procedure

Chicks of at least 1 week of age were considered sufficiently independent to remain unattended in a respiration chamber for 24 h. Validation experiments were performed on chicks of 1 (broilers: $n = 6$, layers: $n = 7$), 2 (broilers: $n = 6$, layers: $n = 5$), and 3 weeks of age (broilers: $n = 6$, layers: $n = 6$). In addition, we performed measurements at sexual maturity (7 weeks of age) on five birds of each strain. Each bird was used once, except for one layer that was used at both 1 and 7 weeks of age.

After measuring body mass (to 0.1 g), the chicks were injected intraperitoneally with a doubly labelled water mixture (enrichment 31.9% ^2H and 62.3% ^{18}O). The amount of mixture injected was determined by weighing the syringe before and after injection to the nearest 1×10^{-4} g on an analytical balance (Mettler H54, Tiel, The Netherlands), and ranged between 1.5 $mg \cdot g^{-1}$ (adults) and 3 $mg \cdot g^{-1}$ (young chicks). We encountered no problems with leakage of small quantities of isotopes following administration. This enabled us to calculate the individual-specific size of the body water pool on the basis of isotope dilution (see below). After administration, the birds were kept individually in cardboard boxes during a period of 1 h for complete equilibration of the injected isotopes with body water. During this interval the birds were not allowed to drink or eat. Exactly 1 h after administration, the birds were weighed again, and the brachial vein was punctured with a sterile needle for blood collection in several 15 µl micropipettes (initial sample), which were flame-sealed immediately. We stored the samples at 5°C till isotope analysis. Next, the birds were placed in airtight metabolic boxes with *ad lib.* water and food from the same source in the home cages. The boxes were placed in a light- and temperature-regulated

metabolic chamber. The light schedule and temperature were identical to the conditions in the home cages. In most cases five birds were measured simultaneously, each in a separate metabolic box (see below). Exactly 24 h after the initial blood sample, the metabolic box was opened. We weighed the birds and took another blood sample (final sample) following the same procedure as described earlier. In each age category, a group of three birds was used for baseline measurements to determine the population-specific background levels for ^{2}H and ^{18}O. We refrained from taking a background sample from each animal prior to the measurement, because pilot experiments had revealed that such a frequent sampling procedure would interfere with the normal weight gain performance of the chick during the measurement in the metabolic box. To circumvent the lack of information on the individual-specific background levels, we applied a relatively high dose of doubly labelled water (see (121) for a discussion on the relevance of background levels). This makes the DLW calculations almost insensitive to minor differences in background levels. The enrichments of the final blood samples for ^{2}H and ^{18}O were at least 670 parts per million (ppm; i.e. 520 ppm excess) and 2960 ppm (i.e. 960 ppm excess), respectively.

Measurement of CO_2 production with infrared gas analysis

Rates of CO_2 production were measured over 24-h periods in an open air flow system (indirect calorimetry). Dry air was pumped through the boxes at rates varying with age (from ca. 25 $L \cdot h^{-1}$ at 1 week to ca. 120 $L \cdot h^{-1}$ at 7 weeks of age) to obtain a difference in the in- and outflowing air of about 0.5% carbon dioxide (i.e. at the level of our gas standard for calibration of the CO_2 analyser). The flow rate was measured on the inlet air with a mass-flow controller (Type 5850E, Brooks) that was calibrated against a soap foam flow meter (Bubble-O-Meter, La Verne CA). The excurrent air was dried over molecular sieves (3 Å, Merck). The carbon dioxide concentration was measured by an infrared gas analyser (BINOS-IR), and the oxygen concentration by a zirconium oxide analyser (S-3A/II Oxygen Analyser, Applied Electrochemistry), both to an accuracy of 0.01%. We calibrated both analysers daily with certified gas standards (AGA, The Netherlands). To verify the O_2 and CO_2 concentrations of the certified gas standard, an interlaboratory comparison was performed with the Respiration Unit of the Agricultural University of Wageningen. We employed six channels simultaneously, using valves to switch between the channels once per minute (washout time 45 s), so that for each channel the values were recorded automatically at 6-min intervals. The reading for the O_2 and CO_2 concentration of the excurrent air for each box was made during the last 10 s of the flushing period. Five channels were used for measurements of respiration gasses. The sixth channel was used for measurement of the inlet air.

Isotope analysis

The isotopic enrichments of the blood samples were determined in triplicate at the Centre for Isotope Research, University of Groningen, as has been described elsewhere (183). In brief, each capillary was first distilled in a vacuum line. After complete cryogenic transfer of the distilled water sample into a quartz vial (placed in

liquid air) 2 ml of CO_2 gas was added. The vial was placed in a thermostatised water bath (Tamson TC 45, Zoetermeer, The Netherlands) at 25°C during 48 h for equilibration of the CO_2 gas with the distilled water sample. Thereafter, the vial was placed in a dry-ice alcohol mixture and CO_2 gas was cryogenically trapped in another quartz vial placed in liquid air. Lastly, the water sample was cryogenically transferred over a uranium oven and the H_2 gas was trapped in a quartz vial with active charcoal. $^2H/^1H$ and $^{18}O/^{16}O$ isotope ratios were determined from H_2 and CO_2 samples, respectively, by isotope ratio mass spectrometry (SIRA 9, Manchester, UK) with dual inlet for reference and sample gas. For each isotope we applied daily internal gas standards at the background level and at high enrichment to estimate the level of cross-contamination between reference and sample channels (Meijer and Neubert, in prep.). In addition, for each batch we applied at least three differently enriched internal water standards that have been calibrated against IAEA standards. These were taken such that they covered the entire enrichment range of the test samples. Each internal standard was measured in quadruplicate.

Calculations

For each bird the total body water volume (TBW; g) was calculated using the principle of isotope dilution for ^{18}O:

$$TBW = Q \times 18.02 \times \frac{(C_d - C_i)}{(C_i - C_b)} \quad \text{(Eqn 1)}$$

where Q is the size of the DLW dose in moles, and C_d and C_i are the ^{18}O concentrations (atom %) of the dose and the initial blood sample, respectively. C_b is the average ^{18}O concentration of the background. Eqn 1 was also used to calculate the TBW value based on ^2H dilution. For this, we took for C_d the ^2H concentration (atom %) of the dose, for C_i the ^2H concentration of the initial blood sample, and for C_b the average ^2H concentration of the background. For each bird, the dilution space ratio ($R_{dilspace}$) was calculated by dividing the TBW value obtained from ^2H dilution by the value obtained from ^{18}O dilution (174).

Fractional turnover rates were calculated for ^2H (k_d; d^{-1}) and ^{18}O (k_o; d^{-1}) with the general equation:

$$k = \frac{1}{t} \times \ln \frac{(C_i - C_b)}{(C_f - C_b)} \quad \text{(Eqn 2)}$$

where C_f is the ^{18}O concentration of the final blood sample, and t is the time in days elapsed between taking the initial and final blood sample.

Rates of water efflux (rH_2O_{eff}; $g \cdot d^{-1}$) were calculated using Eqn 4 of (122) corrected for fractionation effects of heavy isotopes (Eqn 32 of (88)). We assumed a fractional evaporative water loss of 0.33 (see results section).

Rates of CO_2 production (rCO_2; $L \cdot d^{-1}$) were calculated with the following general equation:

$$rCO_2 = 22.4 \times [\frac{N}{2.078} \times (k_o - k_d) - r_G \times 0.0249 N k_d] \quad \text{(Eqn 3)}$$

where N is the size of the body water pool as assessed from ^{18}O dilution in moles, and r_G is the fraction of the water flux lost through evaporative pathways. This is basically Eqn 34 of (88), but with the most recent estimates for the different fractionation processes following (174). Unfortunately, it is virtually impossible to measure rates of evaporative water loss in animals that have free access to water and food. Therefore, to investigate the relative importance of the assumptions concerning fractional evaporative water loss for each bird, rCO_2 values were calculated at r_G values of 0 (no evaporative water loss as proposed by (121)), 0.25 (as proposed by (174)), 0.50 (as proposed by (88)) 0.75, and 1 (all water is lost through evaporative pathways, i.e. no defecation).

In addition, rCO_2 values were calculated with a two pool model (Eqn 7.43 of (174)) by taking the average $R_{dilspace}$ obtained for the entire data set (see results section). For this model we also investigated the effect of the assumed fractional evaporative water loss on the relative error of the DLW method.

Data analysis

The method of respiration gas analysis to measure rCO_2 (rCO_{2IR}) was considered as the 'golden standard'. Thus, relative errors (%) in rCO_2 values obtained from DLW measurements (rCO_{2DLW}) were calculated as

$$error = 100 \times \frac{(rCO_{2DLW} - rCO_{2IR})}{rCO_{2IR}} \quad \text{(Eqn 4)}$$

Analysis of covariance (ANCOVA) was used to investigate effects of body mass, age, strain, and relative rate of body mass gain of the chick on the relative error of the DLW method (SPSS Inc., 1988). Data are expressed as means and inter-individual standard errors unless stated otherwise. All tests were two-tailed, and significance was accepted at $p < 0.05$.

RESULTS

Weight gain

In table 1 we listed various characteristics of the two strains at the ages measured. For each strain, 18 measurements were performed on growing chicks and five on birds that had achieved sexual maturity (Table 1). In layers two females produced an egg during the measurement. Until 3 weeks of age, the average absolute rates of body mass gain ($g \cdot d^{-1}$) were highest in broilers, ranging from 7.9 $g \cdot d^{-1}$ to 9.4 $g \cdot d^{-1}$. In layers values ranged from 6.4 $g \cdot d^{-1}$ to 8.0 $g \cdot d^{-1}$ (Table 1). Relative rates of weight gain ($\% \cdot d^{-1}$) during the measurement decreased from 18.7 $\% \cdot d^{-1}$ at 1 week of age to 4.5 $\% \cdot d^{-1}$ at 3 weeks of age in broilers. Corresponding values for layers were 16.0 $\% \cdot d^{-1}$ and

Table 1. Body mass (g), absolute ($g \cdot d^{-1}$) and relative rate of body mass gain ($\% \cdot d^{-1}$), total body water volume (TBW; %), rate of water efflux (rH_2O_{eff}; $g \cdot d^{-1}$), rate of CO_2 production measured by respiration gas analysis (rCO_{2IR}; $L \cdot d^{-1}$) and DLW method (rCO_{2DLW}; $L \cdot d^{-1}$; Eqn 6), and the relative error of the DLW method (%) in relation to age (weeks) in two strains of Japanese quail. Values are means ± SDs

age (weeks)	body mass (g)	body mass gain ($g \cdot d^{-1}$)	body mass gain ($\% \cdot d^{-1}$)	TBW† (%)	rH_2O_{eff} ($g \cdot d^{-1}$)	rCO_{2IR} ($L \cdot d^{-1}$)	rCO_{2DLW} ($L \cdot d^{-1}$)	error (%)
broilers‡								
1	48 ± 6.5	8.7 ± 2.2	19 ± 4.4	79 ± 1.5	21 ± 3.4	4.2 ± 0.6	4.3 ± 0.6	1.5 ± 10
2	102 ± 8.6	9.4 ± 5.6	9.0 ± 4.9	77 ± 0.8	31 ± 3.5	7.2 ± 0.8	7.5 ± 0.7	4.5 ± 7.4
3	155 ± 26	7.9 ± 8.4	4.5 ± 5.7	76 ± 1.3	27 ± 7.3	8.5 ± 1.7	8.3 ± 1.7	-1.7 ± 5.5
7	294 ± 41	8.7 ± 6.8	3.2 ± 2.5	67 ± 6.2	61 ± 9.7	12 ± 1.2	12 ± 1.3	-2.2 ± 10
layers								
1	40 ± 6.6	6.4 ± 0.8	16 ± 1.7	80 ± 1.9	18 ± 2.2	3.7 ± 0.5	3.8 ± 0.3	4.2 ± 6.2
2	72 ± 8.9	8.0 ± 0.5	11 ± 1.6	78 ± 0.6	24 ± 3.2	6.1 ± 0.6	5.8 ± 0.9	-6.2 ± 6.7
3	122 ± 17	7.3 ± 2.6	6.1 ± 2.3	76 ± 1.2	29 ± 6.6	7.4 ± 1.0	7.0 ± 0.5	-4.7 ± 11
7	184 ± 13	-6.8 ± 12	-3.5 ± 6.0	62 ± 3.4	40 ± 19	8.2 ± 1.8	8.4 ± 1.9	2.9 ± 4.4

† Based on the dilution space of ^{18}O.
‡ $n = 5$ for broilers of 7 and layers of 2 and 7 weeks of age; $n = 6$ for broilers of 1, 2, and 3 weeks, and layers of 3 weeks of age; $n = 7$ for layers of 1 week of age.

6.1% $\cdot d^{-1}$, respectively (Table 1). In adult animals, broilers showed a slight increase in body mass (g) during the measurement and layers a slight decrease (Table 1).

Rates of CO_2 production: single pool model

As a first step in the analysis, we calculated for each DLW measurement the rate of CO_2 production ($L \cdot d^{-1}$) at each of the assumed levels of fractional evaporative water loss (from 0 to 1; Eqn 3). Next, we determined for each value the error in the rate of CO_2 production relative to respiration gas analysis (%; Eqn 4). We then calculated for the entire data set (both broilers and layers) the mean relative error values for each level of fractional evaporative water loss (Fig. 1). For these mean values the relationship between the relative error of the DLW method (error; %) and the assumed fractional evaporative water loss (r_G) can be approximated by linear regression as

$$\text{error} = 5.2 - 15.8 \times r_G \quad \text{(Eqn 5)}$$

Assuming no fractional evaporative water loss ($r_G = 0$) or a fraction of 0.25, the DLW method tended to overestimate the true rate of CO_2 production by 5.2 ± 1.3% and 1.3 ± 1.3%, respectively ($n = 46$; Fig. 1). Assuming fractions of 0.5, 0.75, or 1, the DLW method tended to underestimate the true rate of CO_2 production by 2.7 ± 1.2%, 6.7 ± 1.2%, and 10.6 ± 1.14%, respectively ($n = 46$). The best fit was obtained at a fraction of 0.33 (Fig. 1). This value was entered in Eqn 3 to yield

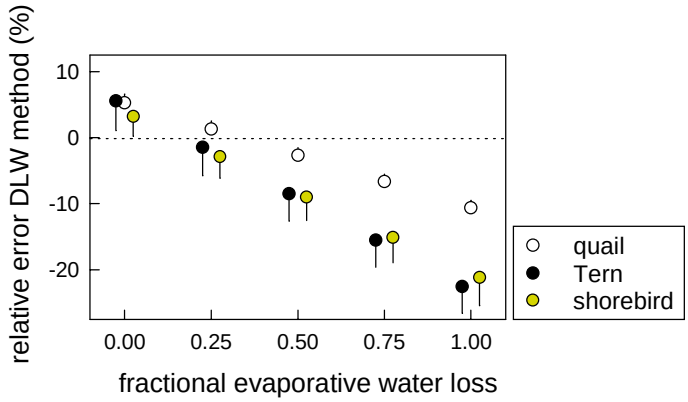

Figure 1. Relationship between mean relative error of the DLW method (%) and assumed fractional evaporative water loss in growing chicks of Japanese quail, Tern, and shorebird. Values are means, and bars indicate SEs.

$$rCO_2 = 22.4 \times [\frac{N}{2.078} \times (k_o - k_d) - 0.00822 \times Nk_d] \quad \text{(Eqn 6)}$$

We used this equation to calculate the rate of CO_2 production and Eqn 4 to calculate the relative error of each DLW estimate to the value obtained with respiration gas analysis. For all individual values the relative errors ranged between –14.4% and 17.3%. The age-specific average errors ranged between –6.2% and 4.5% (Table 1). The relative errors of the rates of CO_2 production in the two females that produced an egg during the measurement were –3.6% and 5.8%, which suggests that Eqn 6 also holds during the egg-laying phase.

Analysis of covariance was used to investigate for both strains the relationship between the relative error of the DLW method and the relative rate of body weight gain during the measurement (observed range from –13.8 $\%\cdot d^{-1}$ to 23.1 $\%\cdot d^{-1}$; Fig. 2). ANCOVA revealed that the interaction term (strain × relative rate of weight gain) did not significantly contribute to the explained variance. After deleting this term from the model, there was no significant effect of strain and relative rate of weight gain during the measurement on the relative error of the DLW method. We performed a similar analysis for the relationship between the age of the chick and the relative error of the DLW method. After deleting the insignificant interaction term (strain × age), no significant effect of both strain and age on the relative error of the DLW method could be detected. We can conclude that Eqn 6 is applicable in birds of both strains over the range of relative weight gain rates observed and ages employed.

Rates of CO_2 production: two pool model

The average value for the dilution space ratio ($R_{dilspace}$) was 1.035 ± 0.0017. Analysis of covariance was used to investigate for both strains the relationship between $R_{dilspace}$ and body mass at the time of injection. The analysis revealed that strain × body mass

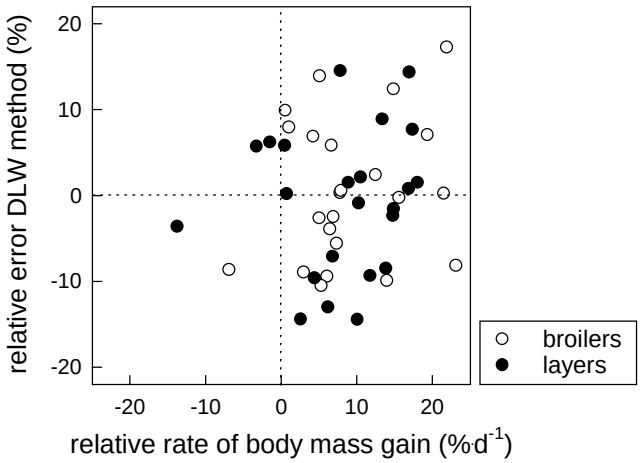

Figure 2. Relative error of the DLW method (%) in relation to relative rate of body mass gain (%·d^{-1}) during the measurement in two strains of Japanese quail at 1, 2, 3, and 7 weeks of age.

interaction did not significantly contribute to the explained variance ($F_{1,42} = 1.7$). After deleting this term from the model, neither strain nor body mass significantly contributed to the explained variance: one value for $R_{dilspace}$ is applicable in chicks of both strains over the range of body masses observed.

Next, we applied the value of 1.035 to calculate the rate of CO_2 production with the two-pool model at fractional evaporative water loss levels of 0, 0.25, and 0.5. The average relative errors of these estimates to respiration gas analysis were –5.7 ± 1.2%, -9.8 ± 1.2%, and –13.9 ± 1.2%, respectively. Thus, at each level of fractional evaporative water loss the two pool model significantly underestimated the true rates of CO_2 production.

DISCUSSION

The experiment was designed to test the applicability of the DLW method in a fast and normal growing strain of Japanese quail, in an attempt to evaluate the occurrence of differential rates of ^2H and ^{18}O incorporation in growing tissues. We demonstrated that in both strains from 1 week of age till the achievement of sexual maturity one single pool model could be used to calculate the rate of CO_2 production from DLW measurements (Eqn 6). This equation is a refinement of Eqn 34 of (88), after making the assumption that a fixed fraction (here estimated as 0.33) of the entire water efflux is lost through evaporative pathways. The fact that one equation could be used over a wide developmental range, and over a wide range of relative rates of weight gain, suggests that differential rates of ^2H and ^{18}O incorporation are unlikely to occur in Japanese quail. At least not at such a level that the application of the DLW method becomes problematic. In fact, relative errors of the DLW method obtained in chicks

are not systematically different from those obtained in adults (including laying females).

Up till now, three studies have been performed to validate the DLW method in growing birds: semi-precocial Arctic Tern ((79); recalculated by (183)), precocial shorebird chicks of the Northern Lapwing and Black-tailed Godwit (183), and Japanese quail (this study). In these studies the same general model was used to calculate rates of CO_2 production (Eqn 34 of (88)). All three studies showed that the DLW method was applicable in growing birds. In none of the studies a significant relationship could be detected between the relative rate of weight gain during the measurement and the relative error of the DLW method. The studies also demonstrated that the error of the DLW method relative to measurements of respiration gas analysis (the 'golden standard') was highly sensitive to assumptions concerning fractional evaporative water loss. In all three studies, the use of a fractional evaporative water loss of 0.50 (as assumed by (88)) resulted in a systematic underestimation of the true rate of CO_2 production (Fig. 1). The largest mean error was made in shorebirds (–9.0%) and Arctic Terns (–8.5%), and the smallest in Japanese quail (–2.7%). Better fits were obtained using Eqn 7.27 of (174) that employs a fraction of 0.25: mean relative errors range from –2.9% in shorebirds to 1.3% in Japanese quail (Fig. 1). On the other hand, neglecting fractionation effects resulted in a systematic overestimation of CO_2 production rate by 3–5% (Fig. 1). Therefore, we can cautiously conclude that using Eqn 7.27 of (174) in growing precocial and semi-precocial birds gives the best estimates if no possibility exists for performing a validation study. It cannot be excluded that fractional evaporative water losses are higher in naked altricial nestlings that lack plumage for insulation.

In the three groups of species mentioned, the slope of the relationship between the assumed fractional evaporative water loss and the relative error of the DLW method was highest in Arctic Tern (-28.1) and lowest in Japanese quail (-15.8; Fig. 1). Thus, the sensitivity of the DLW method differs considerably with respect to assumptions concerning fractional evaporative water loss. It has been argued on the basis of theoretical modelling that this sensitivity strongly depends on rates of water efflux relative to rates of CO_2 production (155). Therefore, in an attempt to explain the differences between the three species, we examined in more detail the rate of CO_2 production ($L \cdot d^{-1}$; from respiration gas analysis), the rate of water efflux ($g \cdot d^{-1}$), and the ratio of water efflux rate relative to CO_2 production rate (water economy index (123); Fig. 3). First, we used analysis of covariance to compare the allometric relationships between rate of CO_2 production and body mass for the different species (Fig. 3A). ANCOVA revealed that the interaction between species and body mass contributed significantly to the explained variance ($F_{2,47} = 26$, $p < 0.001$). The lowest value for the allometric scaling exponent was found in Japanese quail (0.62) and the highest in shorebirds (1.10; Fig. 3A). Secondly, a similar analysis was performed to compare the allometric relationships between water efflux rate and body mass. After deleting the insignificant interaction term (species × body mass), both species ($F_{2,49} = 38$, $p < 0.001$) and body mass ($F_{1,49} = 63$, $p < 0.001$) contributed significantly to the explained variance (Fig. 3B). The common allometric scaling exponent was 0.45 ± 0.057 and the intercept values for Japanese quail, shorebirds, and Terns were 3.34,

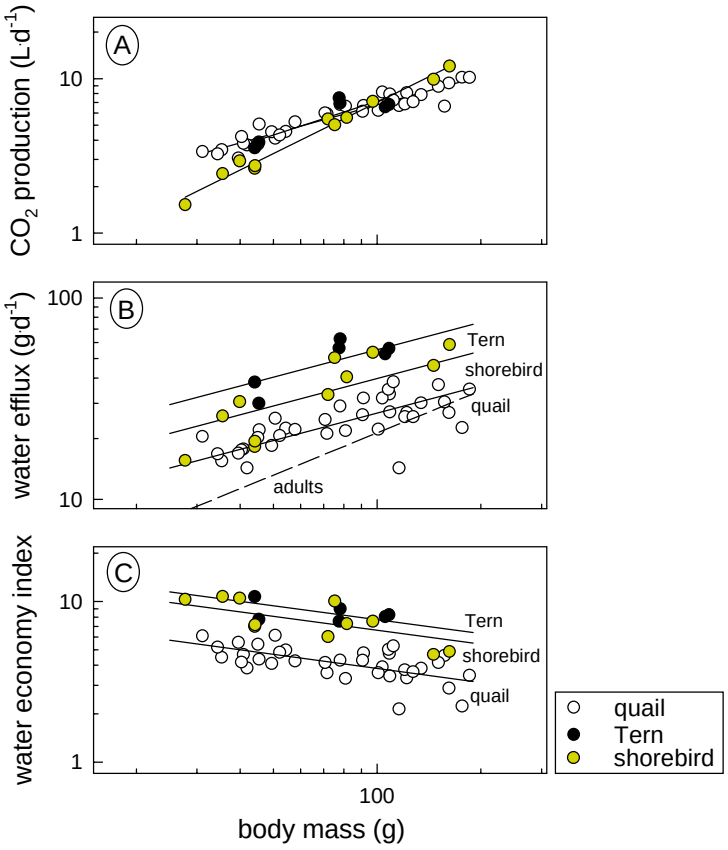

Figure 3. Allometric relationships between CO_2 production rate ($L \cdot d^{-1}$; A), water efflux rate ($g \cdot d^{-1}$; B), and water economy index (C) as a function of body mass (g) in growing chicks of Japanese quail, Tern, and shorebird. Drawn lines refer to the fitted equations for each species. (B) also shows the allometric relationship between body mass and water efflux rate in captive adult birds (123).

4.96, and 6.91, respectively (Fig. 3B). At a given body mass, water fluxes were lowest in Japanese quail, and values were higher in shorebirds and Terns by 48% and 107%, respectively. In nearly all cases observed levels were well above the levels predicted on the basis of an allometric relationship for adult birds in captivity (123). Interestingly, both shorebird and quail chicks were fed a pellet diet, while water fluxes were considerably higher in shorebird chicks. Tern chicks were fed a fish diet. Thirdly, we performed a similar analysis to compare the allometric relationships between index of water economy and body mass (Fig. 3C). After deleting the insignificant interaction term (species × body mass), both species ($F_{2,49} = 58$, $p < 0.001$) and body mass ($F_{1,49} = 33$, $p < 0.001$) contributed significantly to the explained variance. After assuming parallel slopes (-0.29 ± 0.051), it was found that at a given body mass water economy index was lowest in quail chicks, and values were higher

in shorebirds and Terns by 72% and 101%, respectively (Fig.3C). There is a striking relationship between the level of water economy index and the sensitivity of the DLW method to assumptions concerning evaporative water loss. On the one hand, water economy index was low in quail, and the DLW method in these birds appeared to be relatively insensitive to assumptions concerning evaporative water loss. On the other hand, water economy index was high in Tern chicks, and the DLW method in these birds appeared to be more sensitive to assumptions concerning evaporative water loss. As indicated in figure 3B, quail and Tern chicks differ especially with respect to their levels of water efflux.

Japanese quail is the first animal species in which the DLW method has been validated over a wide developmental range. No information is available on other species for comparison. In humans, validation studies have been performed over an even broader range of developmental stages, from pre-term and term babies to adults at normal and high working rates (for the most recent review see (174)). Results on humans showed that one general model (Eqn A6 of (165)) is applicable at all stages. In contrast to the model used by us, Eqn A6 of (165) is based on two assumptions: (1) rate of breath water loss (which is subject to fractionation) is directly related to rate of CO_2 production, and (2) rate of transcutaneous water loss through the skin (also subject to fractionation) is directly related to the surface area of the naked skin. Due to differences in the respiratory system and the level of skin protection between birds and mammals, it is difficult to make a direct comparison between both models (see also the discussion on this issue in (174)). Yet it is encouraging that in both humans and animals the application of the DLW method in juveniles does not appear to be invalidated by the partial incorporation of ^{2}H and ^{18}O in growing tissues.

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SAMENVATTING

Daglengte beïnvloedt de snelheid waarmee jonge vogels en zoogdieren groeien. De meeste dieren zijn overdag of 's nachts actief. De lengte van de dag zal daardoor bepalen hoeveel voedsel (= energie) de dieren gedurende een etmaal kunnen opnemen en hoeveel energie zij zullen uitgeven (die dus niet beschikbaar is voor de groei). Groei is de resultante van deze twee processen, waarop daglengte, variërend bijvoorbeeld per seizoen of breedtegraad, een belangrijke invloed kan uitoefenen. Bijvoorbeeld bij dagactieve dieren zal een langere daglengte meer kansen creëren op voedselopname wat een positief effect heeft op de groei. Langere daglengtes verhogen tevens de totale lichamelijke activiteit wat tot een hogere energie uitgave leidt. Dit beïnvloedt de groei negatief. Korte daglengtes hebben het tegenovergestelde effect, namelijk zowel een lagere opname als een lagere uitgave van energie. Deze interactie tussen de opname en de uitgave van energie als functie van daglengte heeft zo een cruciale invloed op het uiteindelijke gewicht dat bereikt zal worden. Bij dieren die 's nachts actief zijn kunnen we tegengestelde effecten verwachten.

De relatie tussen daglengte en gewichtstoename kan belangrijke gevolgen hebben voor onze kennis over het aanpassingsvermogen van dieren die zich voortplanten op verschillende breedtegraden of tijden van het jaar. Tevens zou deze relatie invloed kunnen hebben op het optimale daglengteschema zoals toegepast in de (pluim)veehouderij. Het is dus belangrijk om inzicht te krijgen in de manier – gedragsmatig en fysiologisch – waarop daglengte de balans tussen de opname en de uitgave van energie, en dus groei, beïnvloedt.

Dieren reageren niet passief op veranderingen in daglengte. Blootgesteld aan kortere daglengtes zijn zij in staat hun eetgedrag en/of lichamelijke activiteiten zodanig aan te passen dat ze hoge groeisnelheden kunnen handhaven. Aanpassingen in de vertering van voedsel is een ander mechanisme waarlangs dieren hoge groeisnelheden kunnen handhaven ondanks kortere daglengtes (en dus kortere periodes met voedsel). Deze aanpassingen kunnen worden bereikt door energie bij voorkeur te gebruiken voor de ontwikkeling van de organen die betrokken zijn bij de voedselvertering en/of via een verbeterde balans tussen voedselopname en vertering. Voedselopname vindt plaats gedurende de dag, terwijl gedurende de nacht, wanneer voedselopname en activiteiten grotendeels afwezig zijn, het grootste deel van de vertering plaatsvindt. Bepaalde licht–donker verhoudingen binnen een etmaal zouden deze balans tussen voedselopname en vertering kunnen verbeteren, en resulteren in hogere groeisnelheden.

Dit proefschrift beschrijft een studie naar de rol van daglengte bij het bepalen van de netto groei. Om dit te bestuderen hebben we gebruik gemaakt van de Japanse kwartel, een dagactief dier met een hoge groeisnelheid en daardoor potentieel zeer gevoelig voor variaties in daglengte. We onderzochten het effect van daglengte op verschillende parameters: opname en uitgave van energie, lichamelijke activiteit, eet- en drinkpatroon, lichaamssamenstelling en eiwitaanmaak. Ter vergelijking hebben we tevens een experiment uitgevoerd naar het effect van daglengte op de groei bij een nachtactief dier, de rat. We kozen voor de rat omdat de gewichtstoename van dit dier gevoelig is gebleken voor verschillen in licht–donker verhoudingen. Verder heeft de rat – als een zoogdier – een lagere groeisnelheid en een lagere energie uitgave dan vogels van hetzelfde gewicht.

Hoofdstuk 2 beschrijft een experiment naar het effect van een lange (18L:6D, oftewel 18 uur licht/6 uur donker) en korte daglengte (6L:18D) op lichamelijke activiteit, lichaamsgewicht, groeiefficiëntie (toename in lichaamsgewicht per gram opgenomen voedsel), voedselopname, energie uitgave en lichaamssamenstelling van jonge ratten. De dieren hadden gedurende het gehele experiment vrij toegang tot voedsel. De resultaten lieten zien dat de gewichtstoename van deze dieren gevoelig was voor daglengte. Dieren met een lange daglengte (voor de rat dus een korte actieve periode) waren aan het einde van het experiment gemiddeld ruim 10% zwaarder dan de dieren met een korte daglengte, ondanks minieme verschillen in voedselopname en energie uitgave. Waarschijnlijk is dit verschil in gewichtstoename het gevolg van verschillen in de verdeling van de energie uitgave, en mogelijk ook van de voedselopname, over het etmaal.

Hoofdstuk 3 beschrijft een experiment naar het effect van verschillende daglengtes (18L:6D, 15L:9D, 12L:12D, 9L:15D en 6L:18D) op de gewichtstoename, voedselopname en energie uitgave van de Japanse kwartel (leeftijd 7–71 dagen). De dieren hadden hierbij uitsluitend tijdens de lichtperiode toegang tot voedsel. Om het effect van daglengte te kunnen onderscheiden van het effect van de beschikbaarheid van voedsel, wordt in dit hoofdstuk een tweede experiment besproken waarbij jonge kwartels werden blootgesteld aan een lange daglengte (18L:6D en 15L:9D) met voedsel uitsluitend gedurende een kort gedeelte (respectievelijk de eerste 6 en 9 uur) van de lichtperiode. Ook bij kwartels bleek de gewichtstoename gevoelig te zijn voor daglengte: langere daglengtes gingen gepaard met een hogere voedselopname en een hogere energie uitgave, resulterend in hogere lichaamsgewichten. De invloed van daglengte op de gewichtstoename was voornamelijk het gevolg van het effect van daglengte op de beschikbaarheid van voedsel. Bij daglengtes van 9 uur en korter was de groei minder vergeleken met langere daglengtes: bij deze daglengtes was de reductie in energie uitgave ten gevolge van een kortere daglengte niet voldoende om de eveneens verlaagde voedselopname te compenseren. De reductie in voedselopname was echter niet evenredig met de afname in daglengte. Kuikens blootgesteld aan daglengtes van 9 uur en korter begonnen al twee dagen na het begin van het experiment voedsel op te slaan in hun krop. Hierdoor waren ze in staat meer voedsel op te nemen dan op basis van de reductie in daglengte kon worden voorspeld. De lagere energie uitgave bij daglengtes van 9 uur en korter was het gevolg van een sterkere verlaging van de energie uitgave gedurende de nacht ten opzichte van de dag vergeleken met daglengtes van 12 uur en langer. Deze aanpassingen in eetgedrag en energie uitgave ten gevolge van daglengte, en dus de beschikbaarheid van voedsel, kunnen gezien worden als onderdeel van een algemene strategie die jonge dieren in staat stelt continu door te kunnen groeien ondanks beperkingen in het voedselaanbod.

Naast aanpassingen van gedrag en energie uitgave kunnen variaties in daglengte ook leiden tot aanpassingen van de organen die betrokken zijn bij de vertering van voedsel. Door een lagere voedselopname bij kortere daglengtes zou het bijvoorbeeld voordelig kunnen zijn om de organen van het maagdarmkanaal, die een relatief hoge energie uitgave hebben, aan te passen aan de verlaagde voedselopname. Dit zou kunnen leiden tot een lagere energie uitgave, waardoor meer energie beschikbaar is

voor groei. In *hoofdstuk 4* wordt het effect van een lange (18L:6D) en korte daglengte (6L:18D) op de ontwikkeling van diverse organen van jonge kwartels (leeftijd van 7–28 dagen) beschreven. De resultaten lieten zien dat jonge kuikens voornamelijk hun krop vergrootten wanneer ze werden blootgesteld aan een korte daglengte. Op deze manier waren ze in staat meer voedsel op te slaan gedurende de periode dat voedsel beschikbaar was. Aanpassingen van de overige organen van het maagdarmkanaal waren niet zichtbaar. Mogelijk is het vergroten van de krop (een metabolisch laag actief weefsel) een relatief goedkope manier om de groei te maximaliseren. Mede doordat door de vergroting van de krop de voedselopname zo sterk toenam dat de overcapaciteit (verhouding tussen de voedselopname en de grootte van een orgaan) van de verteringsorganen teniet werd gedaan. Tevens zou een groot maagdarmkanaal bij lagere voedselopnames de verblijftijd van het voedsel in het maagdarmkanaal kunnen verhogen, hetgeen een positief effect zou kunnen hebben op de verteringsëffientie en dus op de netto groei.

De resultaten, zoals beschreven in de hoofdstukken 3 en 4, lieten zien dat Japanse kwartel kuikens, blootgesteld aan daglengtes van 9 uur en korter, hun krop gingen benutten als tijdelijke opslagplaats voor voedsel. In *hoofdstuk 5* wordt een studie beschreven naar de motivatie hierachter. Japanse kwartel kuikens (leeftijd van 7–31 dagen) werden blootgesteld aan een lange daglengte (18L:6D), waarbij de dieren gedurende een periode van 6 uur toegang hadden tot voedsel. Bij één groep kwam het voedsel dagelijks beschikbaar op een vast tijdstip en bij een tweede groep op willekeurige tijden (in de ochtend, middag of avond). De dieren bleken niet hun krop te vullen in anticipatie op een lange periode zonder voedsel. De variaties in voedselopname waren consistent met een simpel regelmechanisme waarin het eetgedrag alleen is aangepast aan de energiebehoefte van het moment: na een korte periode zonder voedsel was de voedselopname laag ondanks het feit dat de daarop volgende periode zonder voedsel lang kon zijn. Dit effect werd beinvloed door het tijdstip waarop het voedsel gedurende de dag arriveerde: voedsel dat laat beschikbaar kwam leidde tot hogere opnames.

Groei van jonge dieren is hoofdzakelijk groei van eiwit. Het eiwitbestand van het lichaam is een dynamisch geheel dat onderhevig is aan een continu proces van eiwitaanmaak en afbraak: de eiwitturnover. Netto groei van eiwit vindt alleen plaats als de aanmaak groter is dan de afbraak. Zoals bleek uit het voorgaande is groei gevoelig voor daglengte. Het is daarom waarschijnlijk dat daglengte, via de tijd die beschikbaar is voor voedselopname en energie uitgave, ook de eiwitturnover beinvloedt door een effect op de eiwitaanmaak en/of afbraak en zo op de netto groei. *Hoofdstuk 6* is gewijd aan dit onderwerp. Japanse kwartel kuikens (leeftijd van 7–21 dagen) werden blootgesteld aan een lange (18L:6D) of korte daglengte (6L:18D) met gedurende de lichtperiode toegang tot voedsel. We bepaalden de fractionele snelheid van de eiwitaanmaak (het percentage eiwitmassa dat per uur wordt aangemaakt) door een grote hoeveelheid gelabeld aminozuur (aminozuur met een afwijkend atoommassa) te injecteren in het lichaam. Na 15 en 30 min werd de hoeveelheid label gemeten die nog vrij aanwezig was en de hoeveelheid die ondertussen ingebouwd was in eiwit. De verhouding hiertussen is een maat voor de fractionele snelheid van de eiwitaanmaak. Op deze manier werd de eiwitaanmaak gemeten in

borstspier, lever en hart aan het einde van de nacht (= periode zonder voedsel) en nadat de dieren aan het begin van de dag twee uur toegang tot voedsel hadden gehad. Daglengte bleek een duidelijk effect te hebben op de eiwitaanmaak. Lange nachtelijke periodes zonder voedsel zorgden ervoor dat de eiwitaanmaak aan het begin van de dag sterk verlaagd was vergeleken met korte nachtelijke periodes zonder voedsel. Toegang tot voedsel bij een korte daglengte leidde daarentegen tot een sterkere stijging in de eiwitaanmaak dan bij een lange daglengte. Deze fluctuaties in eiwitaanmaak over het etmaal als functie van daglengte (en beschikbaarheid van voedsel) bepalen de snelheid waarmee jonge dieren bij verschillende daglengtes kunnen groeien.

Tenslotte is *hoofdstuk 7* gewijd aan de validatie van de dubbelgelabeld water (DLW; water met verhoogde concentraties van de isotopen ^{2}H en ^{18}O) methode om de energie uitgave te meten bij jonge snelgroeiende dieren. De DLW methode is een techniek om de energie uitgave in de natuurlijke omgeving van het dier te meten, waarbij gelabelde waterstof en zuurstof worden ingespoten in de bloedbaan. Deze methode is gebaseerd op de veronderstelling dat deze labels het lichaamswater alleen verlaten als water (betreft ^{2}H and ^{18}O) en kooldioxide (alleen ^{18}O). Uit het verschil in snelheid waarmee beide labels het lichaamswater verlaten wordt de CO_2 productie (en dus de energie uitgave) berekend. Bij volwassen dieren is deze methode uitgebreid gevalideerd, maar nog nauwelijks bij groeiende dieren. Een probleem dat kan optreden bij groeiende dieren is dat de labels het lichaamswater niet alleen via water of kooldioxide verlaten, maar tevens via inbouw in groeiende weefsels. Dit zou theoretisch kunnen leiden tot een onderschatting van de CO_2 productie. In hoofdstuk 7 beschrijven we een experiment waarin we de resultaten van de DLW methode vergeleken met die van een methode waarbij de CO_2 productie rechtstreeks werd gemeten via analyse van de respiratie gassen (infrarood gasanalyse). Dit werd gedaan bij de Japanse kwartel vanaf de leeftijd van 1 week tot volwassenheid. De resultaten lieten zien dat de DLW methode tevens een goede methode is om de energie uitgave van jonge snelgroeiende vogels te meten.

Samenvattend kunnen we stellen dat daglengte invloed heeft op verschillende aspecten van de groei en dat dit effect voornamelijk wordt bewerkstelligd door de invloed van daglengte op de tijd die per etmaal beschikbaar is voor voedselopname en energie uitgave. We hebben tevens laten zien dat dieren deze variaties in daglengte en voedselbeschikbaarheid niet passief ondergaan. Door verschillende aanpassingen – gedragsmatig en fysiologisch – zijn zij in staat hoge groeisnelheden te behouden ondanks beperkingen in voedselaanbod.

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